ANGLIA RUSKIN UNIVERSITY

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TEAR DYNAMICS: TEAR OSMOLARITY AND REFLEX FEATURES OF THE LACRIMAL FUNCTIONAL UNIT

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ABSTRACT

The focus of this thesis is tear dynamics and the regulation of tear secretion by the Lacrimal Functional Unit (LFU), a collection of functions that control tear secretion and maintain a healthy ocular surface.

Tear secretion was assessed with the Schirmer test and tear osmolarity (tOsm), in standardised environmental conditions achieved using a controlled environment chamber (CEC). The experiments involved normal and dry eye disease (DED) subjects, including Sjögren Syndrome DED.

A novel approach to sheathing the Schirmer strip was tested for the first time in a regulated range of relative humidities achieved in the CEC. This was designed to highlight an artefact of measurement, and free the test from its dependence on ambient conditions.

A period of evaporative suppression, achieved innovatively by eye closure, was shown to drive tOsm down, in normal and DED subjects, to a basal level that fell within the range of plasma osmolarity. This basal tear osmolarity value could be used as a non-invasive measure of systemic dehydration, particularly of use in a care home setting and as a reference against which to gauge the severity of DED in an individual.

Using the sheathed Schirmer strip the existence of 'cross-connections' in humans was investigated for the first time using unilateral anaesthesia in an attempt to answer the question of whether afferent corneal inputs from one eye may have some influence on the lacrimal output of the fellow eye. Previously only studies involving animals had reported positive findings in this area. The relative contributions of each eye to the reflex tear response are relevant in unilateral trigeminal sensory loss cases since sensory input from the healthy eye could reinforce tear secretion of the affected fellow eye.

Lastly, an experiment was designed to strengthen our understanding of the mechanism of the LFU in providing a compensatory response in desiccating conditions. This approach differed from previous studies as it included both normal and DED subjects and involved creating a sensory blockade using topical anaesthesia to increase the stress level experienced by the LFU.

Key words: Lacrimal Functional Unit; Basal Tear Osmolarity; Sensory cross-connectivity; Sheathed Schirmer; Tear osmolarity; Plasma osmolality.

TABLE OF CONTENTS

	Page number
Acknowledgements	i
Abstract	ii
Table of contents	iii
List of figures	vii
List of tables	xii
Abbreviations	xiv
List of appendices	xvi
Copyright declaration	xvii
Chapter 1: INTRODUCTION	1
1.1 The ocular surface	1
1.2 The tears	2
1.2.1 Tear compartments	2
1.2.2 The tear film	2
1.2.3 The precorneal tear film	3
1.2.4 Sources of the tears	3
1.2.5 Composition of the tears	3
1.2.6 Contribution of the tear film to vision	4
1.3 The lacrimal gland	4
1.3.1 Lacrimal secretions	5
1.4 The meibomian gland	5
1.4.1 Meibomian lipids: composition and secretion	6
1.5 The conjunctiva	7
1.5.1 Conjunctival secretions	7
1.6 The cornea	9
1.6.1 Staining of the corneal epithelium	9
1.7 Osmolarity and Osmolality	10
1.7.1 Tonicity	10
1.7.2 Tear hyperosmolarity	10
1.8 The lacrimal functional unit	11
1.8.1 Afferent and efferent signalling	11
1.8.2 Loss of sensory drive	12
1.9 Factors influencing tear osmolarity	13
1.9.1 Tear film lipid layer	13
1.9.2 Palpebral aperture width	13

1.9.3 Blink interval 14
1.9.4 Tear film break-up 15
1.9.5 Effect of ambient humidity 15
1.10 Closed eye tears 19
1.10.1 Increasing relative humidity 19
1.11 Tear osmolarity in the normal eye and dry eye disease 20
1.11.1 Variation and inter-eye difference in tear osmolarity 20
1.12 Diurnal variation in tear osmolarity 21
1.13 Osmolarity of the fluids that comprise the tears 22
1.14 Tear osmolarity and plasma osmolality 22
1.15 Body hydration and dehydration 23
1.16 Tear osmolarity as an index of plasma osmolality and hydration status 25
1.17 Dry eye disease 26
1.17.1 Sub-groups of dry eye disease 26
1.17.2 Aqueous deficient dry eye 27
1.17.3 Evaporative dry eye 28
1.17.4 Hybrid forms of dry eye disease 28
1.18 Vicious circle of dry eye disease 29
1.19 Prevalence and burden of dry eye disease 30
1.20 Quality of life 31
1.21 Aims of the research 31
Chapter 2: METHODOLOGY 34
2.1 Ethical approval 34
2.2 Participants for all studies 34
2.3 Inclusion criteria 34
2.3.1 Normal subjects 34
2.3.2 Dry eye disease patients 35
2.4 Exclusion criteria 35
2.4.1 Normal Subjects 35
2.4.2 Dry eye disease patients 35
2.5 Further classification of dry eye disease patients 36
2.6 Apparatus 36

2.6.1 Controlled environment chamber 36
2.6.2 Slit-lamp 39
2.7 Clinical assessments 39
2.7.1 Clinical history 40
2.7.2 Visual acuity 40
2.7.3 OSDI 40
2.7.4 Tear osmolarity 40
2.7.5 Fluorescein instillation 45
2.7.6 Tear break-up time 45
2.7.7 The Schirmer test 46
2.7.8 Meibography 48
2.7.9 Meibomian gland expression 49
2.7.10 Topical anaestheisa 49
Chapter 3: PILOT STUDY OF THE DEPTH AND DURATION OF TOPICAL ANAESTHESIA AND THE EFFECTS OF ENHANCED DESICCATING STRESS 51
3.1 Pilot study: Depth and Duration of topical anaesthesia 51
3.2 Pilot study: Increased desiccating stress with sensory blockade 52
Chapter 4: STANDARDISING THE SCHIRMER TEST BY ENCLOSING THE STRIP IN A WATERPROOF SHEATH 55
4.1Introduction 55
4.2 Hypothesis 57
4.3 Materials and methods 58
4.3.1 Subject enrolment 58
4.3.2 Equipment 58
4.3.3 Protocol 60
4.3.4 Statistical analysis 62
4.4 Results 62
4.4.1 Slit-lamp examination 70
4.5 Discussion 71
Chapter 5: ESTIMATING BASAL TEAR OSMOLARITY IN NORMAL AND DRY EYE SUBJECTS 77
5.1 Introduction 77
5.2 Hypothesis 79

5.3 Materials and methods 79
5.3.1 Subject enrolment 79
5.3.2 Equipment 80
5.3.3 Protocol 81
5.3.4 Statistical analysis 82
5.4 Results 83
5.5 Discussion 101
Chapter 6: CENTRAL CONNECTIONS OF THE LACRIMAL FUNCTIONAL UNIT 107
6.1 Introduction 107
6.2 Hypothesis 110
6.3 Material and methods 110
6.3.1 Subject enrolment 110
6.3.2 Equipment 111
6.3.3 Protocol 113
6.3.4 Statistical analysis 115
6.4 Results 115
6.5 Discussion 117
Chapter 7: THE EFFECT OF DESICCATING STRESS ON TEAR OSMOLARITY WITH AND WITHOUT SENSORY BLOCKADE 124
7.1 Introduction 124
7.2 Hypothesis 125
7.3 Material and methods 125
7.3.1 Subject enrolment 125
7.3.2 Equipment 126
7.3.3 Protocol 127
7.3.4 Statistical analysis 128
7.4 Results 129
7.5 Discussion 141
Chapter 8: CONCLUSION 145
References 152
Appendices 188

LIST OF FIGURES

Page number

Figure 1.1 The relationship between environmental humidity and tear film evaporation rate from the ocular surface (TEROS). From Tsubota and Yamada (1992). *Invest Ophthalmol Vis Sci* **33**(10): 2942-2950. 16

Figure 1.2 Vicious circle theory of DED pathology. From the Pathophysiology report of TFOS DEWS II. Bron et al. (2017). *Ocul Surf* **15**(3): 438-510. 27

Figure 2.1 A scattergraph showing the timescale of equilibration of the CEC once turned on (at time 0), set to 20ºC and 10% RH. The CEC door was opened at time points of 65 and 70 minutes (Data collected 14th January 2014). 37

Figure 2.2 Dimensions and layout of the CEC. 38

Figure 2.3 Subject sitting in the CEC facing the laminar airflow. 38

Figure 2.4 Subject sitting in the CEC for a slit-lamp examination. 39

Figure 2.5 TearLab® disposable test card, displaying channel that collects tear meniscus sample. 44

Figure 2.6 Tearlab® pens and docking station. 44

Figure 2.7 Sampling of the tears from the meniscus with the TearLab® osmometer. 45

Figure 2.8 Unsheathed Biotech® Schirmer strip, with arrow showing where the strip is folded before insertion. 48

Figure 3.1 Fluorescein staining images of the right and left eye following instillation of anaesthetics and pre- and post-exposure to enhanced desiccating stress. 54

Figure 4.1 Wetting curves obtained for Black ribbon and Whatman 41 filter paper in free absorption. From Holly et al. (1982). *Curr Eye Res* **2**(1): 57-70). 57

Figure 4.2 A Biotech® Schirmer strip, an unbonded, porous paper strip 35mm by 5mm (Whatman No. 41). 59

Figure 4.3 A Biotech® Schirmer strip, enclosed in the lightweight sheaths were constructed from plastic sheeting. 59

Figure 4.4 Images of how the Schirmer strip was inserted into the sheath before use on a subject. This was completed approximated 10 minutes before use. New sheaths were created for each subject. 60

Figure 4.5 Timeline of visit. 61

Figure 4.6 Boxplot showing the Schirmer wetting length in the sheathed and unsheathed conditions at 5% RH. 63

Figure 4.7 Stacked bar graph showing the Schirmer wetting length in 63

the sheathed and unsheathed conditions of each participant at 5% RH.
Figure 4.8 Boxplot showing the Schirmer wetting length in the sheathed and unsheathed conditions at 15% RH. 65
Figure 4.9 Stacked bar graph showing the Schirmer wetting length in the sheathed and unsheathed conditions of each participant at 15% RH. 65
Figure 4.10 Boxplot showing the Schirmer wetting length in the sheathed and unsheathed conditions at 25% RH. 67
Figure 4.11 Stacked bar graph showing the Schirmer wetting length in the sheathed and unsheathed conditions of each participant at 25% RH. 67
Figure 4.12 Boxplot showing the Schirmer wetting length in the sheathed and unsheathed conditions at 45% RH. 69
Figure 4.13 Stacked bar graph showing the Schirmer wetting length in the sheathed and unsheathed conditions of each participant at 45% RH. 69
Figure 4.14 Bar graph demonstrating the mean differences in Schirmer wetting length between the sheathed and unsheathed conditions across four different exposure levels. 70
Figure 4.15 Examples of typical fluorescein staining of the palpebral conjuntiva following a sheathed and unsheathed Schirmer test. 71
Figure 4.16 Position of a sheathed and unsheathed Schirmer strip once in situ. 74
Figure 4.17 Schematic of a Schirmer strip inserted into the tear lake shown in cross section as an equilateral triangle. Curved lines with arrows indicate tear evaporation. From Telles et al. (2017). *Colloids Surf Physicochem Eng Aspects* **521**:61-68. 75
Figure 5.1 Timeline of eye closure and exposure to 70% RH visits. 82
Figure 5.2 Bar graph displaying mean tOsm values for normal and DED subjects (right eye) after 45 minutes of eye closure and then every 15 minutes with eyes open (exposure to 45% RH). 89
Figure 5.3 Bar graph displaying mean tOsm values for normal and DED subjects (left eye) after 45 minutes of eye closure and then every 15 minutes with eyes open (exposure to 45% RH). 90
Figure 5.4 Scattergraph displaying mean tOsm values for normal and DED subjects (right eye) after 45 minutes of eye closure and then every 15 minutes with eyes open (exposure to 45% RH). 90
Figure 5.5 Scattergraph displaying mean tOsm values for DED patients 91

(right eye) after 45 minutes of eye closure and then every 15 minutes with eyes open (exposure to 45% RH). Lines highlight the tOsm values in clinic conditions of individuals and the subsequent levels of tOsm achieved following eye closure.

Figure 5.6 Scattergraph displaying mean tOsm values for normal and DED subjects (left eye) after 45 minutes of eye closure and then every 15 minutes with eyes open (exposure to 45% RH). 91

Figure 5.7 Scattergraph displaying mean tOsm values for DED patients (left eye) after 45 minutes of eye closure and then every 15 minutes with eyes open (exposure to 45% RH). Lines highlight the tOsm values in clinic conditions of individuals and the subsequent levels of tOsm achieved following eye closure. 92

Figure 5.8 Bar graph displaying mean tOsm values for normal and DED subjects (right eye) every 15 minutes with eyes open (exposure to 70% RH). 93

Figure 5.9 Bar graph displaying mean tOsm values for normal and DED subjects (left eye) every 15 minutes with eyes open (exposure to 70% RH). 93

Figure 5.10 Scattergraph displaying mean tOsm values for normal and DED subjects (right eye) every 15 minutes with eyes open (exposure to 70% RH). 94

Figure 5.11 Scattergraph displaying mean tOsm values for normal and DED subjects (left eye) every 15 minutes with eyes open (exposure to 70% RH). 94

Figure 5.12 Bar graph displaying mean tOsm values for normal and DED (both eyes averaged) subjects after 45 minutes of eye closure and then every 15 minutes with eyes open (exposure to 45% RH). 95

Figure 5.13 Scattergraph displaying mean tOsm values for normal and DED subjects (both eyes averaged) after 45 minutes of eye closure and then every 15 minutes with eyes open (exposure to 45% RH). 96

Figure 5.14 Scattergraph displaying mean tOsm values for DED patients (both eyes) after 45 minutes of eye closure and then every 15 minutes with eyes open (exposure to 45% RH). Lines highlight the tOsm values in clinic conditions of individuals and the subsequent levels of tOsm achieved following eye closure. 96

Figure 5.15 Bar graph displaying mean tOsm values for normal and DED subjects (both eyes averaged) every 15 minutes with eyes open (exposure to 70% RH). 97

Figure 5.16 Scattergraph displaying mean tOsm values for normal and DED subjects (both eyes averaged) every 15 minutes with eyes open (exposure to 70% RH). 98

Figure 5.17 Bar graph displaying mean tOsm values for normal and DED subjects (right eye) in clinic conditions, after 45 minutes exposure to 70% RH and immediately after eye opening. 99

Figure 5.18 Bar graph displaying mean tOsm values for normal and DED subjects (left eye) in clinic conditions, after 45 minutes exposure to 70% RH and immediately after eye opening. 99

Figure 5.19 Bar graph displaying mean tOsm values for normal and DED subjects (both eyes averaged) in clinic conditions, after 45 minutes exposure to 70% RH and immediately after eye opening. 100

Figure 6.1 A schematic of the neural reflex arc and possible cross-sensory innervation input from the ipsilateral and contralateral afferents resulting in lacrimal gland stimulation. Additional inputs to lacrimal secretion include (clockwise from top left) skin, emotion, nasal mucosa, and retina. 108

Figure 6.2 Timeline of bilateral saline, bilateral anaesthetics or unilateral saline and anaesthetic visits. 114

Figure 6.3 Schirmer wetting test scores for all 8 participants, after bilateral saline, unilateral anaesthesia, and bilateral anaesthesia phases. 117

Figure 7.1 Timeline of anaesthetic and saline visits. 128

Figure 7.2 Tear osmolarity in normal subjects following instillation of topical anaesthetic in clinic conditions and at 20 minutes time points during exposure to ‘desiccating conditions’ for 60 minutes. 131

Figure 7.3 Tear osmolarity in normal subjects following instillation of topical saline in clinic conditions and at 20 minutes time points during exposure to ‘desiccating conditions’ for 60 minutes. 132

Figure 7.4 Tear osmolarity in DED subjects following instillation of topical anaesthetic in clinic conditions and at 20 minutes time points during exposure to ‘desiccating conditions’ for 60 minutes. 134

Figure 7.5 Tear osmolarity in DED subjects following instillation of topical saline in clinic conditions and at 20 minutes time points during exposure to ‘desiccating conditions’ for 60 minutes. 135

Figure 7.6 Slit-lamp images with fluorescein pre and post CEC exposure to desiccating conditions for 60 minutes and after anaesthetic instillation in normal subjects 1-4. 137

Figure 7.7 Slit-lamp images with fluorescein pre and post CEC exposure to desiccating conditions for 60 minutes and after anaesthetic instillation in normal subjects 5-8. 138

Figure 7.8 Slit-lamp images with fluorescein pre and post CEC exposure to desiccating conditions for 60 minutes and after anaesthetic instillation in DED subjects 1-4. 139

Figure 7.9 Slit-lamp images with fluorescein pre and post CEC exposure to desiccating conditions for 60 minutes and after anaesthetic instillation in DED patients 5-8. 140

All error bars on figures represent confidence intervals (95%) unless otherwise stated.

LIST OF TABLES

	Page number
Table 1.1 Average tOsm values for normal subjects.	20
Table 2.1 Non-invasive Meibography grading scale. From Arita et al. (2013). *Cornea* **32**(3): 242-247.	49
Table 2.2 Grading of Meibomian expression. From Foulks and Bron, (2003). *The ocul surf* **1**(3): 107-126.	49
Table 3.1 Corneal sensitivity before anaesthesia and measured at five minute intervals for 45 minutes after anaesthesia.	52
Table 4.1 Sheathed and unsheathed wetting length results for each participant in 5% RH.	58
Table 4.2 Sheathed and unsheathed wetting length results for each participant in 15% RH.	62
Table 4.3 Sheathed and unsheathed wetting length results for each participant in 25% RH.	64
Table 4.4 Sheathed and unsheathed wetting length results for each participant in 45% RH.	66
Table 4.5 Sheathed and unsheathed wetting length results for each participant at 45% RH.	68
Table 5.1 Mean tOsm over an 8 hour period. From Niimi et al. (2013). *Cornea* **32**(10): 1305-1310.	77
Table 5.2 Profiles of the DED and normal subjects at the screening visit.	80
Table 5.3 Defined environmental conditions used in the current study.	80
Table 5.4 Temperature and RH levels recorded in the uncontrolled, clinic conditions.	84
Table 5.5 Tear osmolarity values for normal subjects following 45 minutes of eye closure and 45 minutes exposure to 45% RH.	85
Table 5.6 Tear osmolarity values for Normal subjects following 45 minutes exposure to 70% RH.	86
Table 5.7 Tear osmolarity values for DED patients following 45 minutes of eye closure and 45 minutes exposure to 45% RH.	87
Table 5.8 Tear osmolarity values for DED patients following 45 minutes exposure to 70% RH.	88
Table 6.1 Profiles of the normal subjects consented for this study.	111
Table 6.2 Summary of experimental design for each subject.	114
Table 6.3 Summary of raw data for all subjects.	116
Table 6.4 Summary of unilateral anaesthesia studies.	122
Table 7.1 Profiles of the normal subjects consented for this study.	126

Table 7.2 Environmental conditions employed in this experiment. 127

Table 7.3 Temperature and RH levels recorded in the uncontrolled clinic conditions. 129

Table 7.4 Data for normal subjects in ‘clinic conditions’ and at three time points during exposure to ‘desiccating conditions’ after instillation of topical anaesthetic. 130

Table 7.5 Data for normal subjects in ‘clinic conditions’ and at three time points with saline (control) instillation and exposure to ‘desiccating conditions’. 132

Table 7.6 Data for DED subjects in ‘clinic conditions’ and at three time points with topical anaesthetic instillation and exposure to ‘desiccating conditions’. 133

Table 7.7 Data for DED subjects in ‘clinic conditions’ and at three time points with saline (control) instillation and exposure to ‘desiccating conditions’. 135

ABBREVIATIONS

ACh	Acetylcholine
ADDE	Aqueous deficient dry eye
AOR	Adjusted odds ratio
AVP	Arginine vasopressin
BE	Both eyes
CEC	Controlled environment chamber
Cl^-	Chloride
DED	Dry eye disease
EDE	Evaporative deficient dry eye
EGF	Epidermal growth factor
HCO_3^-	Bicarbonate
HSK	Herpes simplex keratits
HZO	Herpes zoster ophthalmicus
K^+	Potassium
LE	Left eye
LFU	Lacrimal functional unit
MAPK	Mitogen activated kinases
MCJs	Mucocutaneous junctions
MGD	Meibomian gland dysfunction
MMP-9	Matrix metalloproteinase-9
N	Normal
Na^+	Sodium
NEI-VFQ	National eye institute visual function questionnaire
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NK	Neurotrophic keratitis
NSDE	Non-Sjögren dry eye
OAHFA	O-acyl-ω-hydroxy-fatty-acid
OPI	Ocular protection index

OSDI	Ocular surface disease index
PED	Persistent epithelial defect
PEK	Punctate epithelial keratitis
PMNs	Polymorphonuclear leukocytes
pOsm	Plasma osmolality
pO_2	Partial pressure of oxygen
PRT	Phenol red thread test
QoL	Quality of life
RE	Right eye
RH	Relative humidity
SAPK	Stress activated kinases
sIgA	Secretory immunoglobulin A
SjS	Sjögrens syndrome
SSDE	Sjögren syndrome dry eye
SSN	Superior salivatory nucleus
TBUT	Tear break-up time
TFLL	Tear film lipid layer
TJs	Tight junctions
tOsm	Tear osmolarity
VA	Visual acuity
VDU	Visual display unit
VIP	Vasoactive intestinal peptide

LIST OF APPENDICES

	Page number
Appendix I: Participant information sheet (Chapter 2)	188
Appendix II: Study consent form (Chapter 2)	192
Appendix III: Letter to GP (Chapter 2)	194
Appendix IV: Ocular Surface Disease Index (OSDI)	195
Appendix V: Oxford corneal staining grading scale	197
Appendix VI: Meibography grading scale	198
Appendix VII: Example recruitment record card (Chapter 2)	199
Appendix VIII: Example record card (Chapter 4)	203
Appendix IX: Example record card (Chapter 5)	204
Appendix X: Example record card (Chapter 6)	206
Appendix XI: Example record card (Chapter 7)	209
Appendix XII: Slit-lamp images (Chapter 7)	211
Figures A12 a-l Fluroscein slit-lamp images of the left eye following anaesthetic instillation and exposure to 60 minutes desiccating conditions and right and left images following saline instillation and exposure to desiccating conditions for 60 minutes for normal and DED subjects.	212
Appendix XIII: The Utility Of The Tearlab® Device As A Laboratory Instrument And In Clinical Use	224
Appendix XIV: Supporting publications	232

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Chapter 1:

INTRODUCTION

The focus of the research presented in this thesis is tear physiology and its relationship to dry eye disease (DED), a common, symptomatic disorder responsible for chronic disability on a global scale. Regardless of aetiology, its key mechanism is tear hyperosmolarity, which causes inflammation and damage to the ocular surface. In the normal eye osmolar homeostasis of the tears is closely regulated by the lacrimal functional Unit (LFU) and the research reported here was directed at the mechanism of this control system and how it is disturbed by DED. An approach was also developed to determine a basal level of tear osmolarity for an individual which can be an index of their body hydration state and also a guide to the severity of dry eye in the presence of tear hyperosmolarity. The subject of DED has been reviewed recently in depth in the Tear Film and Ocular Surface Dry Eye WorkShop II report (Nelson et al., 2017) of particular interest are the chapters on pathophysiology, the tear film and epidemiology.

1.1 The ocular surface

The ocular surface is a mucous membrane, lined by a continuous sheet of epithelium, which covers the cornea, the anterior globe and tarsi and extends peripherally to the mucocutaneous junctions (MCJs) of the lid margins. Gipson (2007) defined an ocular surface system, which includes the epithelial surface and glandular epithelia of the cornea, conjunctiva, lacrimal and meibomian glands, their apical (tears) and basal (connective tissue) matrices, the eyelashes with their associated glands, those components of the eyelids responsible for the blink and finally, the nasolacrimal duct. “All components of the system are linked functionally by continuity of the epithelia, and by the endocrine, vascular, and immune systems” (Gipson, 2007).

The ocular surface is one of the most exposed mucosal surfaces in the body, and the ocular surface system ensures that it is wetted at all times, despite constant exposure to a desiccating environment. This ensures that the vital, refractive corneal surface is always covered by a protective and optically smooth tear film. Like other mucosal tissues the ocular surface houses an immune response system capable of defending against microbial and other insults (Knop and Knop, 2002; Streilein, 2003; Dong et al., 2011; Barabino et al., 2012).

Maintenance of homeostasis in response to an ever-changing external environment is achieved by adjustments to the tear flow on a moment to moment basis, and is an important requirement for ocular surface health. Disruption of this closely integrated

system can have system-wide consequences that are relevant to the multifactorial condition of DED (Bron et al., 2017).

1.2 The tears

The tears serve a multitude of functions for the ocular surface providing nourishment, lubrication, immune defence and, above all, an optically smooth, precorneal surface, critical for retinal image formation and stable vision (Reiger, 1992; Montés-Micó, Alió, and Charman, 2004; Denoyer, Rabut and Baudouin, 2012). Maintenance of the tear film is a constant task, a balance between the production, evaporation and drainage of tears (Tsubota, 1998).

1.2.1 Tear compartments

In the open eye the tears are distributed between three compartments: the cul-de-sac (fornix and retrotarsal space), the preocular tear film and the menisci (Yokoi et al., 2004). The preocular tears form a layer over the exposed cornea and conjunctiva and therefore have precorneal and prebulbar elements. The upper and lower tear menisci, formed by surface tension forces in the upstroke of the blink, lie in the groove between the globe and lid margins. The peripheral apices of the menisci are located just behind the MCJs of the lid margins while their central apices are found at zones of meniscus–induced thinning, seen as 'black lines' on fluorescein staining, which separate the menisci from the tear film, (Miller, Polse and Radke, 2002). The menisci provide conduits to the lacrimal puncta and nasolacrimal system necessary for tear drainage. It is thought that the tear osmolarity (tOsm) is not distributed uniformly across these three compartments, for instance, based on modelling considerations, meniscus osmolarity is predicted to be lower than tear film osmolarity (Gaffney et al., 2010). Not only this, but an osmolarity gradient within the meniscus itself is thought to exist, being at its highest at the apex where it makes contact with the MCJs and is purported to be the basis of Marx's line; epithelial cells that stain with lissamine green (Bron et al., 2011a; b).

1.2.2 The tear film

Hydration of the ocular surface is maintained by the tears, which bathe it continuously and provide an unbroken film over its exposed surface. The open eye is constantly subjected to desiccating stress through evaporation of the tears, but is protected from damage by homeostatic mechanisms that regulate tear secretion and distribution in response to signals from the ocular surface. In DED, a failure of these mechanisms leads to a quantitative or qualitative deficiency of tears that typically induces tear film instability, wetting defects and hyperosmolar stress, increased friction and chronic mechanical irritation at the ocular surface. This initiates a chain of inflammatory events and surface

damage that characterise the disease.

1.2.3 The precorneal tear film

Wolff's (1946) early concept of the precorneal tear film was of a three-layered structure consisting of a superficial lipid layer, an aqueous layer and a deep mucin layer (Wolff, 1946) and this is still a reasonable approximation of the current view. Holly and Lemp (1971) later proposed that the mucin of goblet cell origin was distributed in two phases, a deep viscous phase associated with the corneal epithelium and a more dilute, aqueous phase interacting with the lipid layer (Holly and Lemp, 1971). This concept of a coacervate, implying an aggregate held together by hydrophobic bonding, is no longer thought to be tenable. Instead it is considered that secretory mucin is present as a distinct but dynamic gel layer, the mucoaqueous subphase, held together by weak non-covalent interactions, hydrogen bonds, Van der Waals attractions and steric interactions, between the polymer molecules (Yokoi, Bron and Georgiev, 2014). This mucoaqueous subphase in turn, is thought to be loosely associated (Cher, 2008) with the water-wettable glycocalyx (Cope et al., 1986) of the surface epithelium. The thickness of the tear film lipid layer (TFLL) is in the region of 15 -157 nm with an average of 42 nm (King-Smith, Hinel and Nichols, 2010) while that of the precorneal tear film, based on evidence from reflection spectra, is about 2-3 μm (King-Smith et al., 2000, 2004; Wang et al., 2003). The tear film thickness over the conjunctiva is not known.

1.2.4 Sources of the tears

The aqueous tears are secreted chiefly by the lacrimal glands, with additional contributions from the conjunctiva, including the goblet cells and a small fraction from the corneal epithelium. The meibomian glands are the source of the TFLL. Using fluorophotometry it has been calculated that the normal tear volume is in the region of 7μL (Mishima, 1965), with the maximum capacity of the conjunctival sac without spillover, about 30μL (Mishima et al., 1966). The secretory rate has been estimated at 1.03 ± 0.39 μl/min with a tear turnover of 16.19 ± 5.10% (Tomlinson, Doane and McFadyen, 2009). Yokoi et al., (2004) using the radius of curvature of the tear meniscus as an index of total tear volume, calculated tear volume to be in the region of 9.6μl in normal subjects and 6.7μL in a group of subjects with subjects with aqueous-deficient dry eye (ADDE) (Yokoi et al., 2004).

1.2.5 Composition of the tears

The aqueous tears contain hundreds of proteins (de Souza, de Godoy and Mann, 2006; Zhou et al., 2012) including maintenance proteins such as epidermal growth factor (EGF) which sustain epithelial cell renewal and defence proteins such as lysozyme, lactoferrin,

and immunoglobulins, involved with adaptive immunity (van Haeringen 1981; Paulsen, 2006). In inflammatory circumstances, plasma proteins (albumin) may diffuse into the tears due to an increased permeability of the conjunctival vessels (Sack, Tan and Tan, 1992) and epithelium (Bron et al., 2017). The chief mucin of mucoaqueous layer is the large gel-forming mucin (MUC5AC) secreted by the goblet cells which plays a role in lubrication and in the removal of cellular and microbial debris from the ocular surface.

1.2.6 Contribution of the tear film to vision

In order to maintain the optical quality of an image transmitted to the retina the tear film must be constantly replenished, since it provides one of the refractive elements in the eye (Craig, Blades and Patel, 1995). Higher order aberrations generated by the tear film in the blink interval can disturb the quality of the retinal image. These aberrations diminish steadily to an optimum level after a blink and then increase again until reset by the next blink cycle. In one report the aberrations reached a minimum at 6.1 ± 0.5 seconds after a blink in normal subjects and at 2.9 ± 0.4 seconds in DED subjects and then rose thereafter (Montés-Micó, Alió, and Charman, 2004).

1.3 The lacrimal gland

The lacrimal gland is a serous gland composed mainly of acinar cells (80%), together with ductal and myoepithelial cells, the combination forming a tubular network (Obata, 2006). The main gland consists of a larger orbital and a smaller palpebral lobe. The orbital ducts fuse with those of the palpebral gland and the lacrimal secretion is delivered into the superior fornix (Fernandez-Valencia and Pellico, 1990) via 6-12 orifices (Bron, 1986). Additionally, there are accessory lacrimal glands, located in the upper and lower fornix, (Wolff, 1946) which form about 10% of the overall tissue mass (Allansmith et al., 1976). The innervation of the accessory glands is similar to that of the main gland (Seifert and Spitznas, 1999) and they are assumed to respond similarly to direct and reflex stimulation. This is in contrast with an earlier view that the accessory glands were responsible for a basal level secretion, such as that measured after topical anaesthesia (Jones, 1966). The lacrimal gland is richly innervated by parasympathetic, sympathetic, and sensory nerves (Burkett et al., 2006). Parasympathetic nerves, containing acetylcholine (ACh) and vasoactive intestinal peptide (VIP), and sympathetic nerves containing norepinephrine, provide a potent stimulus to lacrimal gland secretion (Hodges and Dartt, 2003) and the gland is influenced additionally by many hormones from the hypothalamic-pituitary-gonadal axis (Sullivan et al., 1998). In humans, the lacrimal gland is regarded as the major source of aqueous tears, probably responsible, in the absence of excessive ocular surface stress, for over 90% of the aqueous tear production. Cerretani and Radke (2014),

in their model of human tear dynamics concluded that the contribution of osmotically-driven water flow to the total tear supply, from the conjunctiva and cornea, was in the region of 10% (Cerretani and Radke, 2014). However, Zhu and Chauhan (2012) have speculated, based on experimental measurements that conjunctival secretion could be as high as 1-2 μL/min and could account for the total resting tear secretion (Zhu and Chauhan, 2007). This question is not resolved. In the resting state, tear production is regulated mainly reflexly in response to afferent impulses from the ocular surface (Jordan and Baum, 1980; Gaffney et al., 2010; Willshire, Buckley and Bron, 2017a) and additionally by inputs from the nasal mucosa (Gupta, Heigle and Pflugfelder, 1997), the retina, pain sources and those from higher centres. This tightly regulated and dynamic system constantly modifies tear flow to maintain a properly hydrated ocular surface at all times under varying environmental conditions. Flow rate increases dramatically in the reflex lacrimatory response to nociceptive stimuli (e.g. to a corneal foreign body), or in emotional tearing, (Murube, 2009) when lacrimal secretion can increase over 100-fold over that in everyday conditions (Jordan and Baum, 1980).

1.3.1 Lacrimal secretions

The lacrimal secretion, derived from the lacrimal acini, is modified as it passes through the lacrimal ducts and its composition differs from that of the lacrimal fluid that is delivered into the conjunctival sac. The duct epithelium adds water and electrolytes, particularly K^+ and Cl^- ions (Dartt, Moller and Poulsen,1981; Mircheff, 1989; Ubels et al., 1994; Dartt 2009; Toth-Molnar et al., 2013). In the rabbit, it has been estimated that the duct cells secrete about 30% of the lacrimal fluid (Toth-Molnar et al., 2013) but the figure for human lacrimal fluid is not known. It will be seen that the term lacrimal fluid is not synonymous with that of the tears. The lacrimal fluid refers to the combined secretion of the lacrimal acini and ducts. To this are added the secretions of the conjunctiva and cornea which are mixed with the lacrimal fluid by blinking (Gaffney et al., 2010) and to a lesser extent by eye movements (Yokoi, Bron and Georgiev, 2014), and further, shed epithelial cells and neutrophils. Thus the tears contain proteins, carbohydrates, lipids and salts, aqueous fluid from three sources, goblet cell mucin and various mucin fragments, proteins, glucose and urea of plasma origin and cells, such as epithelial cells and polymorphonuclear leukocytes (PMNs), with TFLL being the major source of lipids. It is this mixture, usually low in cell content in normal eyes that is sampled from the lower tear meniscus for tOsm measurements. Importantly the osmolarity of the lacrimal fluid cannot be assumed to be the same as that of the tears.

1.4 The meibomian glands

The meibomian glands are holocrine, tubulo-acinar, sebaceous glands whose acini discharge their whole contents in the process of secretion. Each gland consists of a series

of grapelike acinar clusters connected by fine ductules to a main duct, lying in parallel rows in the upper and lower tarsal plates (Foulks and Bron, 2003). There are about 25 glands in the upper eyelid and 20 in the lower eyelid (Obata, 2002). The meibomian glands are surrounded by a rich network of nerve endings that derive from parasympathetic (mainly cholinergic but also VIP-ergic), sympathetic and sensory fibres. Parasympathetic nerve terminals have been found in close contact with the basal lamina of the meibomian acini (Knop et al., 2011). The homologous sebaceous glands of Moll and Zeiss, present on the lid margin, also receive a parasympathetic innervation (LeDoux et al., 2001). The rate of meibomian lipid synthesis is regulated by androgen sex steroids (Perra et al., 1990; Sullivan et al., 1998) and the rate at which acinar cells are ruptured and disgorge their contents is controlled by the release of neurotransmitters from the nerves surrounding the acini (Chung, Tigges and Stone, 1996); both can both influence secretion.

Meibomian oil (or meibum) consists of a mixture of lipids which have a melting range between 19.5–32.9°C and is liquid at lid temperature (Tiffany, 1987). Core lid temperature is in the region of 37°C. Delivery of oil to the lid margin is due in part to a steady secretory process and in part to the delivery of small aliquots with each blink (Linton, Curnow and Riley, 1961; Chew et al., 1993; Bron and Tiffany, 1998). The lipid is secreted into a shallow reservoir on the lid margin skin, via orifices situated just anterior to the MCJs. From here, it is delivered to the TFLL in the upstroke of the blink (King-Smith et al., 2004; Bron et al., 2014) spreading rapidly over the aqueous layer and stabilising within 1-2 seconds after the onset of the blink (Bron et al., 2004; Yokoi et al., 2008).

1.4.1 Meibomian lipids-composition and secretion

The meibomian lipids consist of a mixture of polar and non-polar lipids. The non- polar lipids make up about 95% of the total and comprise mainly wax and cholesterol esters, with a small amount of triglycerides. The remainder consists of polar lipids, particularly the amphipathic lipid, O-acyl-ω-hydroxy-fatty acid (OAHFA), the major surfactant of the tears, and a small amount of phospholipids (Butovich, 2009; Brown et al., 2016). The relative amount of phospholipid compared to OAHFA is greater in the TFLL. Other components of meibum include hydrocarbons and free fatty acids (Tiffany and Marsden, 1982; Dougherty and McCulley, 1986; Bron and Tiffany, 1998; Willcox et al., 2017).

The thickness of the TFLL varies regionally and at various stages of the blink interval but averages at around 42 nm (King-Smith, Hinel and Nichols, 2010). Holly (1973), and later McCulley and Shine (1997) proposed that it is a biphasic structure comprising a deep amphiphilic layer of polar lipids, capable of interacting with the aqueous phase of the tear film and a thicker, superficial, lipophilic, layer of non-polar lipids, in contact with the air (Holly, 1973; McCulley and Shine, 1997). It was envisaged that spreading of the TFLL,

driven by surface tension forces, is initiated by movement of the polar elements of the lipid layer over the aqueous phase of the tear film, while the non-polar lipids follow closely behind, interacting with the non-polar hydrocarbon tails of the polar lipids. Lipocalins of lacrimal gland origin, the dominant lipid-binding proteins of the tears, help to stabilise the tear film by scavenging lipids from the mucoaqueous phase and by interacting with the polar lipids of the TFLL to reduce surface tension (Nagyova and Tiffany, 1999). This is important for the spreading of the tears over the ocular surface. The TFLL plays an important role in stabilising the tear film and in the past was thought to play a direct role as a barrier to water loss from the eye, retarding evaporation by up to 90% (Tiffany, 1987; Bron and Tiffany, 2004; Knop et al., 2011). However, recent experiments with meibomian lipid *in vitro* suggest that this figure is more like 10%. The fact remains that in the clinical situation, a TFLL deficiency is associated with an increased evaporative loss (Craig and Tomlinson, 1997) and so it is surmised that the TFLL plays its role in retarding tear evaporation in part by its effect as a lipid barrier and in part stabilising the tear film (Willcox et al., 2017).

1.5 The conjunctiva

The conjunctiva is a transparent mucous membrane that lines the inner surface of the eyelids and covers the anterior surface of the eyeball. It exhibits three distinct regions, palpebral, fornical and bulbar, all of which are moistened by the tears and act as a protective barrier against the external environment (Bron et al., 2004; Knop and Knop, 2005). Intercellular spaces between the conjunctival epithelium are thought to play a role in water transport across the epithelium (Bron et al., 2015). Goblet cells are found throughout the conjunctiva except for a small patch just outside the temporal limbus (Kessing, 1966). Their numbers are greatest in the inferonasal bulbar conjunctiva. Goblet cell, gel mucin (Argüeso and Gipson, 2001) has a high water-binding capacity that facilitates the creation of the mucoaqueous phase of the tear film the major contributor to its thickness (Mantelli and Argüeso, 2008). Conjunctival innervation is by the ophthalmic division (V_1) of the trigeminal nerve, similar to that of the eyelids.

1.5.1 Conjunctival secretions

The mucous membrane of the conjunctiva extends from the corneal limbus, over the anterior globe, into the fornices, across the tarsal plates and ends at the MCJs of the lid margins. The strips of mucosa that form the posterior border of the lid margin are overlain by the tear menisci (Bron et al., 2011a). The conjunctival epithelium consists of two cell types, stratified squamous and goblet cells, which together secrete water, electrolytes, mucins and proteins into the tears (Dartt, 2002; Schmidt et al., 2013). The most superficial, layer 1, cells of the epithelium are highly specialised, being connected to

neighbouring cells by occludens type cell junctions (tight junctions –TJs) and expressing the transmembrane mucins, MUC1, MUC4 and MUC16 at the apices of their epithelial microvilli. The exodomains of these mucins, extending as far as 500nm into the mucoaqueous layer of the tear film (MUC16) and crosslinked with galectin-3 (Argueso et al., 2009), form the glycocalyx of the epithelium which, together with that of the cornea, confers wettability on the ocular surface epithelium as a whole (Cope et al., 1986). The mucins of the glycocalyx interacting with the gel mucins of the tears, anchor the tear film to the ocular surface (Gipson, Hori and Argüeso, 2004). MUC16, with galectin-3, in particular, is responsible for the exclusion of small molecules such as dyes, into the healthy epithelium and, with the TJs of layer 1, are responsible barrier properties of the conjunctival epithelium overall. The permeability of the conjunctival epithelium is 15-25 times greater than that of the cornea (Hämäläinen et al. 1997) probably because of differences in the functional state of certain junctional proteins, such as the claudins (Yoshida, Ban and Kinoshita, 2009).

Goblet cells are the source of the secretory, gel-forming mucin, MUC5AC which binds with the aqueous portion of the tears to form the mucoaqueous subphase of the tear film, (Argüeso and Gipson, 2001). Functions of this secretory mucin include water-binding, lubrication and the trapping of inflammatory and shed epithelial cells and of microorganisms, inhibiting their attachment to the epithelium. Gel mucin also binds sIgA and several antimicrobial proteins and peptides (Gordon, Romanowski and McDermott, 2005). Mucin components found in the tears are a mixture of secreted and shed membrane-associated mucins (Spurr-Michaud *et al.* 2007). The role of the mucoaqueous gel and of the epithelial glycocalyx in reducing friction between the lids and globe during the blink cycle and eye movements has been summarised by Pult et al., (2015) and Bron et al., (2017) (Pult et al., 2015; Bron et al., 2017). Loss of this lubricative function is a source of cell shedding and symptoms in DED. When two apposed surfaces move in relation to each other, the degree of friction depends on the nature of the surfaces, the speed of movement, load applied and presence of lubrication. Hydrodynamic lubrication occurs where surfaces are separated by a fluid layer and depends on the thickness of the layer and its physical properties. Tears are a viscoelastic fluid and in the normal eye, bulk tear fluid is supplied mainly by lacrimal and some conjunctival secretion. Goblet cell mucin imparts a non-Newtonian property to the mucoaqueous phase, so that, with increasing shear rate, as in the blink and saccade, the tear film shear-thins and effective lubrication is achieved. Boundary lubrication applies when the relative motion is slow and there is minimal fluid between apposed surfaces. For the ocular surface, this is when the eyes are almost stationary, or for the lids, probably at the return points of the blink cycle (Pult et al., 2015). In these circumstances, the mucin exodomains of the healthy glycocalyx act as hydrophilic polymer brushes, which greatly lower friction between the lids and globe (Bielecki and Dohan Ehrenfest, 2012; Bielecki et al., 2013) and minimise frictional damage

(Johnson and Murphy, 2004). The action of the glycocalyx is further discussed by Sumiyoshi et al., (2008) and Cai and Wei (2013) (Sumiyoshi et al., 2008; Cai and Wei, 2013). Dartt (2002) summarised the neural and other factors influencing goblet cell and stratified conjunctival epithelial secretion (Dartt, 2002). Nerve terminals are found in proximity to but not in direct contact with the goblet cells and the stratified squamous epithelial cells receive sensory nerve endings. Dartt concluded that parasympathetic nerves stimulate goblet, but not stratified squamous, cell secretion. Sympathetic nerves stimulate stratified squamous, but not goblet, cell secretion, while purinergic (P2Y2), agonists stimulate secretion from both cell types (Dartt, 2002). Growth factors also regulate goblet cell secretion.

1.6 The cornea

The cornea is an avascular, transparent tissue that consists of the epithelium, Bowman's layer, stroma, the Descemet's and pre-Descemet's layers and the endothelium. The cornea has a convex outer surface that provides the majority of the refractive power of the eye and the smooth quality of this optical surface is maintained by the overlying tear film. As in the conjunctiva, the layer 1 cells of the corneal epithelium are connected by TJs, which, here, confer the properties of an almost perfect semi-permeable membrane (Maurice, 1968). The TJs together with the epithelial glycocalyx form a barrier to the passage of ions and of water-soluble molecules from the tears into the cornea. As noted, a similar barrier within the conjunctival epithelium is 15-25 times more permeable than that of the cornea. The glycocalyx of the corneal epithelium plays a similar role in relation to the tear film as that of the conjunctiva, providing an anchoring system for the mucoaqueous layer. The ATP-dependent transport of chloride by the corneal epithelium, from stroma to tears, is associated with the movement of fluid into the tears, thereby contributing to its composition. This contributes too, in a small way, to corneal deturgescence, achieved mainly by active transport of bicarbonate ions from stroma to aqueous, by the corneal endothelium (Klyce and Crosson, 1985).

1.6.1 Staining of the corneal epithelium

The existence of ocular surface punctate staining is used extensively in the diagnosis and management of DED and the distribution of micropunctate staining may provide information as to the cause of damage. Corneal and conjunctival staining provides information about the severity of DED status, but the level of correlation with the mild/moderate DED state is poor (Sullivan et al., 2010). Normal corneas show a low level of punctate epithelial stain (Norn, 1970) attributed to the uptake of fluorescein dye into surface epithelial cells prior to shedding, after loss of their glycocalyx barrier (Bron et al.,

2015). In DED there is, in addition, a failure of the TJs barriers, so that dye can diffuse into the inter or paracellular spaces to gain access to and stain the cells.

1.7 Osmolarity and Osmolality

The ocular surface is constantly bathed in tears whose composition is controlled to ensure a healthy environment for the epithelium. The terms osmolarity and osmolality are used to describe the concentration of dissolved solute particles within a solution such as the tears. Each ion, such as Na^+ and Cl^- counts as a separate particle. The term *osmolarity* describes the number of osmoles of solute per litre of solution while the term *osmolality* refers to the number of osmoles per kilogram of solution. Osmolarity is affected by temperature while osmolality is not. Of relevance to the research presented in this thesis, the term osmolarity is used with reference to tears, expressed as mOsm/L, whereas osmolality is used when referring to plasma, expressed as mOsm/kg. The term osmolarity is also used with reference to serum or plasma when the value is calculated from the concentration of selected solutes such as, Na^+, K^+, glucose and urea osmolarity (Hooper et al., 2015). The numerical difference between the two terms is small and is not generally of clinical significance. In this thesis the terms are used, where possible, according to the context.

1.7.1 Tonicity

The term *tonicity* differs from that of osmolarity or osmolality. It refers to the concentration of *osmotically active* particles in a solution and therefore, for the tears, on the concentration of charged particles, mainly ions, such as the cations, sodium, potassium and calcium and the anions, chloride, bicarbonate and phosphate. Proteins, which are charged, and glucose, which is not, are present in the tears at low concentration and therefore make only a small contribution to tonicity (Murube, 2006). Tear glucose can contribute about 0.2mOsm/L to tear osmolarity (Sen and Sarin, 1980). Urea, a permeate molecule which passes readily through cell membranes, is present in tears at a concentration almost identical to that of plasma, e.g. around 6 mOsm/L (Gavrilov et al., 2000).

1.7.2 Tear hyperosmolarity

Tear hyperosmolarity is a cardinal feature of DED and implies an elevation of osmolarity above that of the tears in normal eyes. Tear hyperosmolarity sets up a series of damaging events at the surface of the eye that will be described later. Hyperosmolar tears can also be designated as hypertonic, implying that the concentration of osmotically active molecules is higher than that of healthy, euhydrated, living cells. Exposure of such cells to

hypertonicity leads to the osmotic removal of water and cell shrinkage, which is one pathway to cell death. Osmometers such as the TearLab® device, based on the measurement of electrical impedence, and hence the presence of charged particles, may be said to be measuring 'effective osmolarity', almost equivalent to tonicity, and do not respond to the presence of urea and glucose in the tears. Therefore the measured osmolarity is likely to be underestimated by approximately 6-7 mOsm/L.

1.8 The Lacrimal Functional Unit

The secretion of tears must be highly regulated to provide a stable tear film and maintain tOsm within narrow limits. When the eyes are open, the ocular surface is constantly exposed to a changing external environment and a physiological system exists that adjusts tear flow to achieve homeostasis. The concept of the lacrimal functional unit (LFU) was put forward as the mechanism responsible for the exquisite control of tear secretion in response to signals from the ocular surface (Stern et al., 1998). The LFU consists of the ocular surface, its secretory appendages and the connecting innervation which reflexly maintains tear homeostasis (Stern et al., 2004). The ocular surface includes the epithelia of the cornea, conjunctiva and lid margins that receive a sensory trigeminal innervation and the secretory appendages are the lacrimal and meibomian glands, the conjunctival goblet cells and the lining epithelia, that receive an efferent, autonomic innervation.

1.8.1 Afferent and efferent signalling

The afferent limb of the reflex arc arises in the peripheral endings of the ophthalmic division of the Vth cranial nerve (the trigeminal nerve - V1), particularly those neurones that supply the cornea. The sensory innervation of the cornea is the highest in the body, with an innervation density about 10-20 times that of dental pulp (Rozsa and Beuerman, 1982). The sensitivity of the posterior lid margin is similar to that of the central cornea (McGowan, Lawrenson and Ruskell, 1994). Centrally, these trigeminal neurones synapse in the superior salivatory nucleus (SSN) in the midbrain (Stern et al., 2004; Meng and Kurose, 2013) with parasympathetic neurones that travel via the *n. intermedius* of the VIIth facial nerve to the pterygopalatine ganglion. Here, these neurones synapse with third order neurones which project to the lacrimal and meibomian glands and to the goblet cells of the conjunctiva. Lacrimal secretion is stimulated by the release of neurotransmitters (Acetyl choline and vasointestinal peptide) from these neurones that modulate protein and water transport (Dartt, 2009). The neurones of the efferent sympathetic pathway, influencing lacrimal secretion to a lesser degree, synapse in the superior cervical ganglion before joining the parasympathetic pathway to innervate the lacrimal gland (Boberg-Ans, 1955;

Stern et al., 1998; Dartt, 2009). Other inputs to the lacrimal gland act via the SSN in conjunction with those from the cornea, from the nasal mucosa, skin (not only pain but also including lid margin and ciliary/lash region -'tickle'), retina (bright lights), acute pain and emotional stimuli. These influence lacrimal secretion from moment to moment and also determine the responsiveness of the gland to other sensory stimuli. Belmonte et al., (2004) have documented the sensory modalities that supply the mammalian cornea, including the human cornea (Belmonte et al., 2004). Of these, 20% are mechano-nociceptors which respond to mechanical forces; 70% are polymodal-nociceptors which respond to extreme temperatures, exogenous irritant chemicals and endogenous inflammatory mediators and 10% are cold receptors, activated by evaporative cooling of the ocular surface (Belmonte and Gallar, 2011). It has been suggested that cold thermoreceptors, which discharge spontaneously at rest, provide the main neural input regulating resting tear secretion. The polymodal-nociceptors have been found to contribute most significantly to the reflex tear secretion (Acosta et al., 2004). Blink rate, too, is inversely related to temperature, a fall in ambient temperature inducing a rise in blink rate (Nakamori et al., 1997).

1.8.2 Loss of sensory drive

A blockade to afferent signals, particularly from the cornea, that renders the ocular surface anaesthetic, can interrupt the feedback loop of the LFU. This can occur with acquired conditions, as well as with anaesthesia induced by topical anaesthetics. Bilateral topical anaesthesia has been shown to reduce but not abolish both lacrimal secretion measured by the Schirmer Ib test (Lamberts, Foster and Perry, 1979; Jordan and Baum, 1980; Li, Deng and He, 2012) and that measured by fluorophotometry (Jordan and Baum, 1980), which confirms that tear secretion is normally maintained, when the eyes are open, not only by a sensory drive from the ocular surface, but an additional contribution from other sources (Lamberts, Foster and Perry, 1979; Li, Deng and He, 2012). This includes both higher centres and the nasal mucosa (Heigle and Pflugfelder, 1996; Gupta, Heigle and Pflugfelder, 1997). Loss of sensory drive to the lacrimal gland also occurs in the trigeminal anaesthesia associated with neurotrophic keratitis (NK) (Heigle and Pflugfelder, 1996). This results in both a reduction of lacrimal secretion, favouring the development of ADDE and a fall in blink rate (Bonini et al., 2003; Sacchetti and Lambiase, 2014), which, by extending the blink interval and increasing evaporative water loss, amplifies its severity. Additionally, removing the trophic support of sensory neurones (Blanco-Mezquita et al., 2013) impairs the maintenance of a healthy ocular surface epithelium and its repair, and renders it vulnerable to external trauma, a situation exacerbated by the loss of the protective blink reflex. The extent to which sensory drive from a fellow, normal eye can compensate for and limit the risk of dry eye on the anaesthetised side is not established. In particular, the nature of any sensory input from a normal cornea to the contralateral

SSN is not known and hence its potential to influence contralateral lacrimal gland secretion is uncertain. It is on this basis that the experiments presented in Chapter 5 were designed, to study the relative contribution of each eye to the reflex tear response, measured by the Schirmer test, in controlled environmental conditions, after unilateral and bilateral topical anaesthesia.

1.9 Factors influencing tear osmolarity

Tear film osmolarity is the central feature of DED pathogenesis and is a function of tear flow, evaporation and drainage (Gilbard and Farris, 1979; Tomlinson, Doane and Mcfadyen, 2009). Water loss, caused by evaporation during the blink interval, creates a thinning of the tear film and concentrates solute, thereby increasing tear osmolarity (Nicholls, Mitchell and King-Smith, 2005). A value of 302 ± 9.7 mOsm/L has been cited as the average tear osmolarity for normal eyes (Tomlinson et al., 2006), and a value as high as 365 ± 77 mOsm/L has been recorded in DED patients, with the depression of freezing point method (Gilbard and Farris, 1979). It is likely that the true levels of osmolarity achieved at the ocular surface in DED are far in excess of this, especially in areas of tear thinning and break up, where “hot spots” of hyperosmolarity are thought to occur (Begley et al., 2006; Liu et al., 2006; Harrison et al., 2008; Peng et al., 2014). These localised regions of increased evaporation also coincide with areas of ocular surface cooling (Li et al., 2015).

1.9.1 Tear film lipid layer

The presence of the lipid layer helps to retard water loss through evaporation. When the tear lipid layer is increased in thickness by meibomian gland expression (Craig, Blades and Patel, 1995) this can result in reduced evaporation in both normal and DED subjects (Arciniega et al., 2011). A qualitative or quantitative deficiency of the lipid layer can amplify evaporation (Craig and Tomlinson, 1997; Borchman, Yappert and Foulks, 2010), and it is likely that this can occur, too, with a deficient aqueous layer, when the spreading of the lipid layer is compromised (Yokoi et al., 2008). Either situation can lead to tear hyperosmolarity.

1.9.2 Palpebral aperture width

The width of the palpebral aperture determines the surface area of eye exposed to evaporative loss. In Caucasians, the average palpebral aperture height in primary gaze, is reported to be 8.3 ± 2.3 mm, equivalent to an exposed surface area of 1.25 cm^2 (Zaman, Doughty and Button,1998). The anatomy of the superior eyelid differs in the Asian population with either the presence (‘double lid’) or absence (‘single lid’) of a superior palpebral crease. A study by Craig et al., (2016) reported the vertical palpebral

aperture as 10 ± 2 mm in Asian subjects with a single eyelid (n=23) and an exposed ocular surface of 2.06 ± 6.2 cm^2 and 11 ± 2 mm in Asian subjects with a double eyelid (n=28) with an exposed surface of 2.40 ± 5.8 cm^2 (Craig et al., 2016). Since gaze position influences aperture width, it also influences water loss from the ocular surface. It was found that the rate of evaporative loss, measured at 40% RH and with a blink rate of 30 per/minute, increased 3.4 times when looking up and 2.5 times when looking straight ahead, when compared to looking down (Tsubota and Nakamori, 1995).

1.9.3 Blink interval

The tear film is refreshed by blinking (Collins, Stahmer and Pearson, 1989). The time between blinks influences tOsm, prolonged exposure increasing the total water loss from the ocular surface. An increased blink interval i.e. a slower blink rate, is a source of desiccating stress, which, in the absence of compensation, should result in increased osmolarity. The blink rate is reduced in response to a variety of factors including environment, visual tasks and behaviour. In healthy adults a blink rate of 14.3 per minute and blink cycle of 4.2 seconds has been reported in standard conditions (Tsubota and Nakamori, 1995). Visual concentration such as that adopted for VDU use (Patel et al., 1991), reading (Ousler et al., 2015) and using hand-held video games (Tsubota and Nakamori, 1993; Jansen et al., 2010) has been found to reduce blink rate, whereas conversation (Doughty, 2001) and DED (Nakamori et al., 1997) are associated with an increased blink rate. Increased wind speed, a source of increased evaporation from and cooling of, the ocular surface, increases the blink rate (Nakamori et al., 1997). The inferior portion of the ocular surface is at risk of remaining exposed to evaporation during an incomplete or partial blink. This occurs when the upper eyelid fails to make contact with the lower lid in the downphase of the blink. In this way on the ascension of the upper eyelid only the tears from the upper meniscus and superior cornea are re-distributed, with the further thinning of the inferior tears a consequence and a hyperosmolarity (McMonnies, 2007). It has been reported that incomplete blinking accounts for between 10-20% of the total number of blinks per minute (Abelson and Holly, 1977; Carney and Hill, 1982). Rogriguez et al., (2013) put forward a further category of ‘rescue blink’ (Rodriguez et al., 2013), a prolonged lid closure considered as a compensatory attempt to increase lipid expression and tear film thickness (Korb et al., 1994). It has also been noted that in a proportion of people there is not always complete contact between the upper and lower lid margins during eye closure, despite a lack of lid pathology. This would result in a potential failure of the eyelids to form an adequate moisture seal and protect against evaporation from the inferior portion of the ocular surface (Blackie and Korb, 2015).

1.9.4 Tear film break up (TBUT)

Evaporation from the ocular surface leads to tear film thinning and instability, resulting in tear film break-up (TBUT), a major contributor to increased tOsm (Foulks, 2007). In the majority of normal eyes the TBUT exceeds that of the blink interval (Lemp and Hamill Jr, 1973), and the tear film is successfully replenished following each blink without disruption of the tear film (Mishima, 1965). Localised thinning of the tear film can occur in areas where the ocular surface wettability is lost (Johnson and Murphy, 2004) and may have pathological effects when it persists with repeated blinks. The relationship between the blink interval and TBUT can be quantified by the ocular protection index (OPI), which is the TBUT divided by the blink interval. When the OPI is <1.0 the ocular surface is exposed to tear break up in the blink interval; the lower the value, the greater the risk of damage through desiccation (Ousler III et al., 2008). When the tear film is formed in the upstroke of the blink, and separates from the tear menisci, it becomes 'perched' and stable in the blink interval (Miller, Polse and Radke, 2002). Tangential flow out of the tear film then makes little contribution to tear film thinning and the major cause of thinning, and hence changes in tOsm, is tear evaporation (King-Smith et al., 2008). Based on tear film thinning rates reported by Nichols et al. (Nichols, Mitchell and King-Smith, 2005) and a tear film thickness of 3µm, it has been calculated that the tear film could thin to zero from the effect of evaporation in approximately nine seconds if not preceded by break up before this time (King-Smith et al., 2008). Hyperosmolarity is amplified in the epithelium in areas of TBUT and extremely high levels of tOsm at the level of the pre-corneal tear film have been predicted mathematically. Based on a thinning of the tear film by a factor of 3 and a baseline tOsm of 300 mOsm/L, it has been calculated that tOsm could be expected to rise to 900 mOsm/L in these local areas of thinning during the blink interval (King-Smith et al., 2008).

1.9.5 Effect of ambient humidity

Evaporation is the transfer of fluid by vaporisation into an unsaturated gaseous phase. For the tears this means the loss of water from the tear film into the ambient air when the relative humidity (RH) is less than 100%. The rate of evaporation from the ocular surface is directly proportional to the ambient humidity (Figure 1.1) (Tsubota and Yamada, 1992). Environmental conditions such as low humidity or increased airflow increase evaporation from the ocular surface (Tsubota and Yamada, 1992; Tsubota, 1998; Uchiyama et al., 2007) and evaporation is increased by higher temperature (Murakami, 2004).

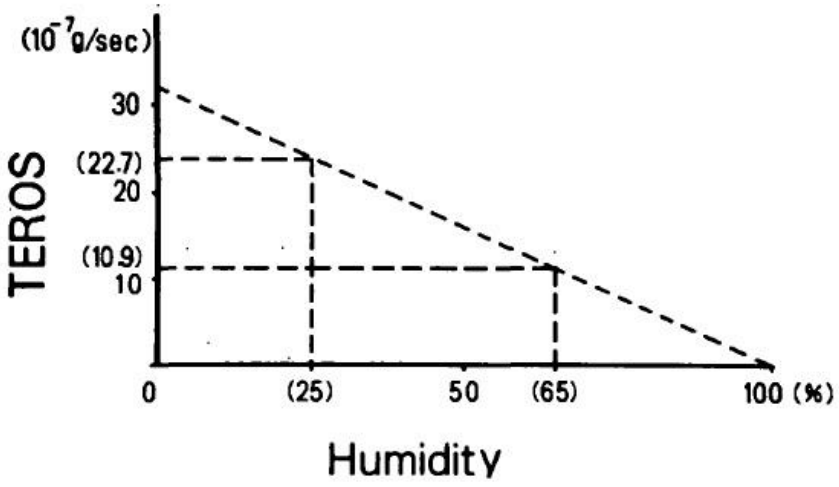


Figure 1.1 The relationship between environmental humidity and tear film evaporation rate from the ocular surface (TEROS). From Tsubota and Yamada (1992). *Invest Ophthalmol Vis Sci* **33**(10): 2942-2950.

A review of the rate of evaporation has been summarised in a paper by Borchman et al., (2009) with figures ranging from 0.0012 to 3.8 µm/min, with average evaporative rates being shown to increase at lower relative humidities (Borchman et al., 2009). At a RH of between 35-45% the evaporative rate was between 0.029 ± 0.009 µL/cm^2/min and 0.043 ± 0.016 µL/cm^2/min, and at RH of between 25-35% the rate was between 0.044 ± 0.013 µL/cm^2/min and 0.058 ± 0.018 µL/cm^2/min (McCulley et al., 2006). In a normal un-stimulated eye it has been found that up to 36% of the tear film is lost through evaporation, compared to 55% in a DED subject (Mathers, 2004), although a more recent study suggests evaporative rates could be at an even higher than this (King-Smith et al., 2008). It is a reasonable prediction that exposure of a subject to a low ambient humidity will result in a rise in tOsm, offset by any compensatory increase in tear flow stimulated by cooling and the rise in osmolarity at the ocular surface. This paradigm has been examined in several studies with some surprising results.

In one study, exposure of normal subjects to prolonged desiccating stress, with an RH of 5%, caused, as expected, a marked increase in tear evaporation compared to that measured at 40% RH, together with symptoms of increased discomfort (Abusharha and Pearce, 2013). Increased discomfort could be explained by surface cooling and a rise in tOsm, due to the increased evaporation. On the other hand, operation of the LFU would be expected to offset these effects of increased evaporation by reflexly stimulating a rise in lacrimal secretion. This would account for the finding that although the average tear osmolarity was higher at 5% RH, it was not significantly different from that at 40% RH. Surprisingly, however, the Schirmer test value at the end of the study, was reduced rather than increased, which conflicted with the expectation of a reflex increase in lacrimal secretion.

In a related study (Teson et al., 2013), DED patients were exposed for two hours, to an environment simulating in-flight airplane cabin conditions (23°C, 5% RH, localised air flow, and reduced barometric pressure of 750 millibars versus 930 millibars at sea level). Patients experienced an increase of symptoms; decrease in TBUT, an increase in corneal staining, a decrease in tear volume measured by the phenol red test (PRT) but an unchanged Schirmer test, measured at 5% RH at the end of the study. Here again, tOsm was not significantly altered.

In a further study by these authors (López-Miguel et al., 2014), a group of DED patients and a group of normal subjects were exposed to the same desiccating environment of 5% RH, for two hours, with increased airflow and a visual task, at normal barometric pressure. The visual task would be expected to bring about a fall in blink rate. This resulted in a shortened TBUT and increased corneal staining and an increase in MMP-9 levels in both groups, but no increase in tOsm in either DED patients or controls; the Schirmer test was increased slightly in the DED group only, and there was no significant increase in symptoms (López-Miguel et al., 2014).

Taken together, these studies support the expectation that prolonged desiccating stress, resulting in increased evaporation of the tears, leads to corneal epithelial damage by reason of presumed, induced tear hyperosmolarity. However, this was not supported by the meniscus tOsm measurements in any of the reports. Several factors may be at play and are the basis of some of the research presented in this thesis.

One possible explanation is that, while tear film hyperosmolarity was the mechanism that induced corneal epithelial damage, insufficient time had elapsed for this local tear hyperosmolarity to be reflected in tear meniscus samples. A potential differential between the meniscus tOsm and tear film tOsm has been predicted from modeling considerations (Gaffney et al., 2010). A means of confirming this would be to measure tissue osmolarity directly at the surface of the cornea (or conjunctiva) during desiccation, but although a device is available (Reis, Grenier and Albuquerque, 2017), its reliability is contested (Rocha et al., 2017).

An additional explanation could be that operation of the LFU resulted in a compensatory increase in tear production, leading to a dilution of the tears which offset the expected rise in meniscus tOsm. If ocular surface damage occurred only in the blink interval during exposure and dilution only occurred with mixing of the tears at the time of the blink, then this would be a reasonable explanation. A means of testing this hypothesis would be to block the afferent limb of the LFU using a topical anaesthetic, in which case it would be predicted that in the absence of compensatory reflex tearing, tOsm would rise in response to the desiccating stress. This forms the basis of the experiments reported in Chapter 6.

Again, if enhanced tear secretion occurred, as anticipated, why was there little evidence of this? In the Teson report (2013), the PRT was reduced and Schirmer test result was unchanged at the end of the study (Teson et al., 2013), and in the Lopez-Miguel report (2014) the PRT test was unchanged and the Schirmer test was slightly increased in the DED group only (López-Miguel et al., 2014). In the Abusharha study the Schirmer test was significantly reduced (Abusharha and Pearce, 2013). One explanation for these results is that they represent an artefact of measurement, caused by a high rate of evaporative water loss from the exposed Schirmer strips at 5% RH. This would explain the apparent reduction in the Schirmer wetting length. Such an effect of low humidity on the wetting length was demonstrated experimentally by Holly and colleagues, who showed that it could be overcome by sheathing the Schirmer paper in a plastic envelope, to prevent evaporative loss (Holly, Lamberts and Esquivel, 1982; Holly, 1994; Beebe, Esquivel and Holly, 1988). This concept is explored in Chapter 3, in which the influence of sheathing on the Schirmer wetting length was studied at a range of different relative humidities, from 5 – 45%. Because this approach is likely to afford a more standardised version of the Schirmer test, sheathed Schirmer strips were used in the study of the central sensory connections of the LFU, in Chapter 5.

Evaporative water loss causes cooling (Craig et al., 2000), which is potentially a source of both symptoms and compensatory tear secretion driven by the activation of thermosensitive corneal cold fibres (Belmonte and Gallar, 2011). Adverse conditions capable of stimulating increased evaporation can be encountered in natural, outdoor environments, or indoors, in the workplace, home and in enclosed places subjected to the influence of air-conditioning.

A study by Khurana et al. (1991) in North India confirmed that dry eye symptoms were more prevalent in rural people exposed to high temperature, low humidity, and dust and hot air currents (Khurana et al., 1991). In the office, complaints of eye discomfort associated with DED were increased in the afternoon (Begley et al., 2002), associated with an increase in temperature and reduction in relative humidity that occurred within the office environment throughout the day (Skov, Valbjørn and Pedersen, 1990). Adverse conditions also exist in aircraft cabins, with levels of RH ranging from 9 to 28% and temperature ranging from 23-24ºC (Nagda and Hodgson, 2001), leading to increased symptoms of DED during flights even in people who were otherwise asymptomatic (McCarty and McCarty, 2000). Additionally 72.3% of pilots were recorded as having an increase in self-reported dryness symptoms following an average of 18.5 hours per week in the low humidity conditions encountered on an aircraft (McCarty and McCarty, 2000).

1.10 Closed eye tears

Eye closure withdraws the ocular surface from evaporative water loss and is equivalent to exposure of the open eye to an RH of 100%, representing total evaporative suppression. Prolonged eye closure, such as that which occurs in sleep, also leads to major changes in the protein composition and cellular content of the tears and a marked reduction in tear flow and drainage compared to that of the open eye (Baum, 1990; Sack et al., 2000). During sleep, special strategies are mounted at the ocular surface that provide an alternative defence against pathogens and an environment of sub-clinical inflammation is created via the up-regulation of immune and inflammatory systems (Sack, Tan and Tan, 1992).

During eye closure there is a fall in pO_2 (partial pressure of oxygen) leading to corneal hypoxia and oedema (Hill and Fatt, 1964; Sarver et al., 1981; Holden, Mertz and McNally 1983) and a fall in tear pH (Carney and Hill, 1976). This situation is reversed within an hour of eye opening when normal aerobic conditions are restored. Sack and colleagues (1992; 2000) demonstrated a significant increase in secretory IgA (rising from 2% to 58%) in closed eye tears and a build-up of tear albumin and other plasma proteins (Sack, Tan and Tan, 1992; Sack et al., 2000), due to vascular leakage (Zantos and Holden, 1978) and enhanced permeability of the conjunctiva. The concentration of proteins of lacrimal acinar origin, such as lactoferrin and lysozyme is markedly reduced, (from 85-88% to 30%) reflecting the fall in lacrimal secretory rate. A massive influx of PMNs occurs in closed eye tears, recruited to the ocular surface by leukotaxic mediators (Lan et al., 1998) and there is activation of the complement pathway (Sack et al., 2000). Although the presence of these PMNs and several of their degranulation products may play a role in defence and the scavenging of micro-organisms, these cells have a refractory phenotype which limits their inflammatory responsiveness (Gorbet, Postnikoff and Williams, 2015).

1.10.1 Increasing relative humidity

Increasing RH, thereby decreasing evaporation from the ocular surface has been used as the basis for a therapy to treat DED. Several studies have been conducted using swimming goggles to reduce evaporation of the tears (Tsubota, 1989; Korb et al., 1996). In these studies, patients with DED reported a reduction in symptoms of ocular discomfort by 99% after 20 minutes wearing the goggles (Korb and Blackie, 2013). The ocular humidity created within the goggles was found to more than double that measured when wearing conventional spectacles, from 34.3 % ± 6.4 to 83.4% ± 5.5 (Tsubota, Yamada and Urayama, 1994). Although the effect was transient, the study demonstrated the benefit of reducing evaporation in maintaining tear film stability.

1.11 Tear Osmolarity in Normal Eyes and in Dry Eye Disease

For the tears, based on a meta-analysis of several studies using depression of freezing point, or vapour pressure measurement, tOsm has been reported to be 302 ± 9.7 mOsm/L in normal adults (Tomlinson et al., 2006). Similar values, using the TearLab® instrument were reported by several other groups, see table 1.1 (Sullivan et al., 2010; Eldridge et al., 2010; Jacobi et al., 2011; Keech, Senchyna and Jones, 2013).

Author	Method	n	tOsm
Eldridge et al., 2010	TearLab®	30	301.8±10.5 mOsms/L
Li et al., 2012	TearLab®	10	298.0±14.2 mOsms/L
Niimi et al., 2012	TearLab®	38	297 ± 15 mOsms/L
Jacobi et al., 2011	TearLab®	133	301mOsmol/L (range 298–304 mOsmol/L)
Sullivan et al., 2010	Tear Lab®	75	302.2 ± 8.3
Keech et al., 2013	Tear Lab®	10 15	304.0 ± 8.4 mOsm/L 301.2 ± 7.2 mOsm/L
Tomlinson et al., 2006	Meta-analysis: Depression of freezing point; vapour pressure; 1978-2005	815	302.0 ± 9.7 mOsm/L

Table 1.1 Average tOsm values for normal subjects.

Tear hyperosmolarity is a key feature of dry eye, values of 322.2 ±18.8 mOsm/L (Sullivan et al., 2010), and 326.9 ± 22.1 mOsm/L (Tomlinson et al., 2006), being reported in populations with mild to severe DED. In the Sullivan report (2010) tOsm in mild/moderate DED was 315 ± 10 mOsm/L and in severe DED, 336 ± 22 mOsm/L (Sullivan et al., 2010). A tOsm of 308mOsm/L is considered to be most sensitive threshold to distinguish normal from mild/moderate forms of DED and 315mOsm/L, the most specific cut off (Lemp et al., 2011).

1.11.1 Variation and Inter-Eye Difference of Tear Osmolarity

Increased variability and inter-eye difference in tOsm have both been shown to be features of DED disease. This departure from the normal state contributes to the pathology of DED and serves as a marker, denoting the breakdown of compensatory mechanisms (Bron *et al.* 2009; Sullivan, Pepose and Foulks, 2015). In the healthy eye tOsm is maintained within a narrow range, reflecting tight homeostatic control by the LFU. Oncel et al. (2012) demonstrated no significant difference in tOsm measured over the course of a day in normal subjects (n=30) (Oncel, Pinarci and Akova, 2012). Conversely several studies have demonstrated increased variability in tOsm measured over a period of time in DED, considered to mirror progression of the disease. Bunya et al. (2015) reported a variability in tOsm over the course of a day of 14.6 mOsm/L in blepharitis patients (n=11) and 15.8 mOsm/L in patients with Sjögren syndrome DED (n=18)

compared to 10.5 mOsm/L in normal subjects (n=18) (Bunya et al., 2015). Similarly, Sullivan et al., (2012) found that variability was significantly increased in severe DED (10 ± 6.9%, n=36) compared to mild/moderate DED (5.9 ± 3.1%, n=16) (Sullivan et al., 2012). In addition the inter-eye difference in tOsm has been reported to increase in parallel with the severity of the disease. Gilbard and Farris (1979) measuring tOsm by a depression of freezing point method, demonstrated an inter-eye difference of 29.7 mOsm/L in *keratoconjunctivitis sicca* subjects (n=30) compared to 6.6 mOsm/L in normal subjects (n=33) (Gilbard and Farris, 1979). Lemp et al., (2011) reported similar a figure, of 6.9 ± 5.9 mOsm/L in normal subjects (n=75), 11.7 ± 10.9 mOsm/L in mild/moderate DED (n=149) and 26.5 ± 22.7 mOsm/L in severe DED (n=75) (Lemp et al., 2011). This variability between the two eyes can be utilised as another diagnostic indicator since it appears to reflect the progression of DED (Lemp et al., 2011; Szalai et al., 2012).

1.12 Diurnal variation of tear osmolarity

Tear osmolarity (tOsm) has been shown to fluctuate diurnally, Terry and Hill (1978) analysed 5µL samples collected from the lower cul-de-sac of six subjects with a thermocouple hygrometer to measures the dew point depression, and reported the average daytime tOsm in 6 young adults to be 310 ± 5.7 mOsm/Kg (range 299-323 mOsm/Kg) (Terry and Hill, 1978), these values overlapping those currently associated with DED (Messmer, Bulgen and Kampik, 2010; Sullivan et al., 2010; Lemp et al., 2011). Terry and Hill (1978) also reported the average daytime tOsm in 6 young adults to be 310 ± 5.7 mOsm/Kg (range 299-323 mOsm/Kg) which compared to a tOsm of 285 ± 2.4 mOsm/Kg (range 282-288 mOsm/Kg), measured immediately after a 6-8 hour period of eye closure (i.e. sleep) (Terry and Hill, 1978). The closed eye values showed less variability and also, it may be noted, but not addressed by the authors, fell within values reported for plasma osmolality (pOsm). This has been measured to range between 285-295 mOsm/Kg in normally hydrated adults (Cheuvront et al., 2010a; Matz, 1996; Stookey, 2005). A similar difference between post-sleep and waking tOsm was reported by Niimi et al., (2013) who found tears to be significantly hypo-osmotic after a period of sleep, 264 ± 14 mOsms/L, compared with the pre-sleep value of 297 ± 15 mOsms/L (Niimi et al., 2013). Thereafter, tOsm rose quickly in the first 10 minutes after eye opening and reached the baseline level within 40 minutes of waking. There was a relatively hyperosmotic trend toward the end of the day.

1.13 Osmolarity of the Fluids that Comprise the Tears

As noted earlier, the tears as sampled at the lower meniscus are composed of a mixture of fluids, mainly of lacrimal origin but also containing conjunctival fluid whose volume contribution is not fully established, plus a small contribution from the cornea. In normal individuals, tOsm is assumed to be higher than that of the secreted tears because of evaporative loss from the tear film (Mishima, 1965; Mishima et al., 1966; Mishima and Maurice, 1961a; b). The osmolarity of the tears is almost entirely dependent on its ionic content and that of a few other small molecules; the contribution of macromolecules such as proteins, in low concentration in the tears (van Haeringen and Glasius, 1977), is small. In both humans (Mircheff, 1989) and rabbits (Dartt, Moller and Poulsen, 1981), the concentrations of Na^+ and HCO_3^- are about the same in both tears and plasma (Krogh, Lund and Pedersen-Bjergaard, 1945; Hind and Goyan, 1949; Thaysen and Thorn, 1954), whereas the concentrations of K^+ and Cl^- are significantly higher in the tears than in the plasma. Thus the composition of the primary secretion of the acinar cells differs from that of the lacrimal fluid delivered into the conjunctival sac. The osmolarity of the conjunctival and corneal secretion is not known.

1.14 Tear and plasma osmolarity and osmolality

There is an historical interest in the measurement of osmolarity in bodily fluids and this is of relevance to the research presented in this thesis. An early study by Krogh et al., (1945) reported that tOsm, measured using a vapour pressure osmometer was ‘similar’ to that of serum in a small group of subjects (308 mOsm/L in tears versus 294.32 mOsm/L in serum) (Krogh, Lund and Pedersen-Bjergaard, 1945). They concluded that the osmolarities of these two fluids might actually be the same, the higher tear value (which would otherwise, nowadays be regarded as indicative of DED), being dependent on evaporation during processing of the tears.

Further evidence of a relationship between pOsm and tOsm was provided recently by Walsh and colleagues (Fortes et al., 2011; Walsh, Fortes and Esmaeelpour, 2011; Walsh et al., 2012) who reported a positive correlation between whole body hydration measured as pOsm, and tOsm, in subjects exposed to systemic dehydration (Fortes et al., 2011). In a study conducted in an environmental chamber, a group of young adults in their twenties were exposed to systemic dehydration, equivalent to 2 to 3% loss of body mass, generated by a combination of water-deprivation and a period of physical exercise. Tear osmolarity followed pOsm closely during the evolution of dehydration and, like pOsm, was restored to normal during rehydration. In this study the pre-exercise pOsm was 288 ± 5 mOsm/kg. In two trials, the mean tOsm correlated strongly with mean pOsm at each time point ($r = 0.93$, $P < 0.001$), suggesting that tOsm could serve as a minimally invasive

surrogate for body hydration of potential use in the rapid detection of dehydration in sports medicine, infants (Bryce et al., 2005) and in the elderly. Fortes et al., (2011) using a tOsm reference value of 310 mOsm/L estimated a sensitivity of 80% and a specificity of 92% using this approach for the detection of suboptimal hydration (Fortes et al., 2011).

In a subsequent study, the authors reported that pOsm may be raised in patients with DED with the implication that the raised tOsm could be a consequence of body dehydration (Walsh, Fortes and Esmaeelpour, 2011). In a following letter they expressed the view that this could lead to a misdiagnosis of dry eye in patients who suffered from systemic dehydration, (Walsh et al., 2012) but Tomlinson et al., (2011) in response, pointed out that the persistent presence of a tear hyperosmolarity within the range consistent with the diagnosis of DED, in conjunction with supportive clinical features, would imply the actual presence of DED (Tomlinson, Madden and Pearce, 2011). Importantly, as noted by Walsh et al., (Walsh, Fortes and Esmaeelpour, 2011), since the risk of both dry eye (Uchino et al., 2006; Moss, Klein and Klein, 2008; Guo et al., 2010) and systemic dehydration (Cheuvront and Kenefick, 2014), increases with age, the value of a raised tOsm in the diagnosis of systemic dehydration is the elderly will be reduced (Walsh, Fortes and Esmaeelpour, 2011; Walsh, et al., 2012; Tomlinson, Madden and Pearce, 2011). It is evident that the occurrence of tear hyperosmolarity due to DED is a potential source of false positives when using tOsm to diagnose systemic dehydration, when based on the results of random, open eye tear samples. However, a way in which this obstacle might be overcome is suggested later.

1.15 Body Hydration and Dehydration

Systemic dehydration is a serious condition, associated with a considerable morbidity and mortality (Xiao, Barber and Campbell, 2004; Manz and Wentz, 2005) which, while unlikely to be overlooked in the hospital population, where serum osmolarity can be readily calculated from blood samples, is underdiagnosed in care-home residents (Wolff, Stuckler and McKee, 2015) and in the elderly population generally. A number of authorities have emphasised the need for a simple, valid, non-invasive screening test that can be used in the community, to diagnose water-loss dehydration in the elderly and aid in its management (US Panel on Dietary Reference Intakes 2004; Cheuvront et al., 2010; Hooper et al., 2015b). The measurement of tOsm is a candidate for the role.

Regulation of water balance is fundamental to survival and is achieved by a combination of renal water conservation and water acquisition in response to thirst. In a hypo-hydrated individual, an increase in pOsm stimulates hypothalamic osmoreceptors and the release of arginine vasopressin (AVP or antidiuretic hormone) from the posterior pituitary. This causes renal water reabsorption, urinary concentration and water conservation

(Cheuvront et al., 2013; Baron et al., 2015). A rise in pOsm also stimulates an increase in water intake in response to thirst (Egan et al., 2003), independent of the action of AVP (Denton et al., 1999; Bourque, 2008).

Systemic dehydration results when loss of body water, with or without salt, occurs at a rate greater than the body can replace it (Thomas et al., 2008). Water-loss is accompanied by plasma hyperosmolarity, plasma hypernatraemia and intracellular dehydration (Cheuvront and Kenefick, 2014). Plasma or serum osmolality, measured directly, or estimated from the chemical composition of these fluids (Hooper et al., 2016, 2015) has long been used as a clinical index of body hydration (Armstrong, 2007; Cheuvront et al., 2010a; Baron et al., 2015), serving as the gold standard against which other less invasive methods are compared in the diagnosis of dehydration. Plasma osmolality in normally hydrated adults measures between 285-295 mOsm/Kg (Matz, 1996; Stookey, 2005; Cheuvront et al., 2010a). Thomas et al., (2008) cite a broader range for serum osmolality of 275 to <295 mOsmol/kg (Thomas et al., 2008). Clinical or 'current' dehydration is defined by a pOsm of >300 mOsm/kg and preclinical, or 'impending' dehydration by a pOsm of >295 or ≤300 mOsm/kg. Note that a level of osmolality that represents clinical dehydration in the body as a whole is compatible with normality at the surface of the eye. The frequency of current dehydration in the elderly population is high, with impending dehydration reported as 40% in those aged 70-90 years, in the US NHANES III cohort, with a further 28% exhibiting current dehydration (pOsm >300mmol/L) (Stookey, 2005). Consequently, dehydration, contributing to the risk of chronic diseases such as urolithiasis, hypertension and coronary heart disease, (Xiao, Barber and Campbell, 2004), is a leading cause of hospitalisation and death in the elderly (Manz and Wentz, 2005; Oei et al., 2016). The risk of dehydration is increased in elderly patients in long-term care. Hooper et al., (2016) reported a frequency of 20% in a population of care home residents (n=188) with a mean age 86 years, with renal, cognitive and diabetic status consistently associated with the risk of dehydration (Hooper et al., 2016). Wolff et al., (2015) in another UK study, basing the diagnosis of dehydration on the presence of hypernatraemia on admission to hospital (plasma Na >145 mmol/L), found a 5-fold increase in the occurrence of dehydration in patients admitted to hospital from care homes (adjusted odds ratio [AOR]: 5.32, 95% CI: 3.85-7.37), compared to that in patients admitted from home, and roughly a two-fold greater risk of in-hospital death (AOR: 1.97, 95% CI: 1.59-2.45) (Wolff, Stuckler and McKee, 2015).

This background emphasises the need to detect dehydration in the elderly, both in the wider community and in individuals in care (Hydration for Health Initiative, 2012). Dehydration is less likely to be overlooked in the hospital population, where serum osmolarity can be readily calculated from blood samples. The osmolalities of other body fluids such as urine and saliva, and urine specific gravity can provide good diagnostic

accuracy under ideal circumstances but are otherwise inferior to pOsm (Cheuvront et al., 2013). Assessment by health or social care workers is more likely to be based on the demonstration of reduced thirst, sense of a dry mouth, furrowing of the tongue, loss of skin turgor, a dry axilla, slow capillary refilling after compression of the nailbed, and increase in urine colour, which appear to be poor indicators of dehydration in older adults (Hooper et al., 2016).

1.16 Tear Osmolarity as an Index of Plasma Osmolality and Hydration Status

As noted, a problem arises in adopting the measurement of tOsm in open eye conditions, to detect sub-optimal body hydration. Tear osmolarity is modified by evaporation and strongly influenced by environmental conditions. Measurement of tOsm in uncontrolled conditions of humidity, temperature and airflow invites variation of tOsm and this effect is amplified in DED (Sullivan, Pepose and Foulks, 2015). Further, since the prevalence of body dehydration rises with age (Xiao, Barber and Campbell, 2004) as does the prevalence of dry eye (Schein et al., 1997; Stapleton et al., 2017), there is an increased likelihood of misdiagnosing systemic dehydration with increasing age; a high tOsm could simply be due to DED and not to dehydration.

A possible approach to overcome this limitation forms the background of the research presented in Chapter 4 of this thesis. It is hypothesised that evaporative suppression, achieved by a suitable period of eye closure or by exposure of the open eyes to an environment of high humidity, will drive down tOsm to a basal level that will be individual to a given person and independent of environmental conditions. It is further predicted that during the period of evaporative suppression, with continued tear turnover and mixing of the lacrimal, conjunctival and corneal fluids, supplemented by equilibration of these fluids across the ocular surface epithelia, particularly the conjunctiva, this basal level tear osmolarity (BTO) will approach that of the plasma (Willshire et al., 2018). This basal tOsm is judged to be individual to the subject and independent of the presence of DED. Chapter 4 reports the effect of a period of either eye closure or exposure to high ambient humidity on tOsm, in a group of subjects with healthy eyes and in a group of patients with DED (Willshire, Buckley and Bron, 2017b).

1.17 Dry eye disease

The research presented in this thesis addresses selected aspects of the LFU, whose dysregulation is a key feature of dry eye. This is recognised in the current definition of DED, reported in the TFOS International Dry Eye Workshop, DEWS II 2017 (Craig et al., 2017) which highlights the homeostatic nature of the ocular system and acknowledges that disturbed neurosensory inputs contribute to the disease. The definition now reads "Dry eye is a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles."

1.17.1 Sub groups of dry eye disease

There are two sub-groups of DED, aqueous-deficient dry eye (ADDE) and evaporative dry eye (EDE), which may occur independently, or in combination, in hybrid forms (Bron et al., 2009, 2017). Regardless of aetiology or subgroup, tear hyperosmolarity is the hallmark of the disease and injures the ocular surface directly or sets in train a chain of signaling events within surface epithelial cells, that leads to the release of inflammatory mediators and proteases. This results in ocular surface inflammation, glycocalyx damage, goblet cell loss and apoptosis of corneal and conjunctival epithelial cells. This series of events is considered to perpetuate the disease, a concept that is embodied as the 'vicious circle' of dry eye (Figure 1.2) (Baudouin, 2007; Baudouin et al., 2013; Lemp et al. DEWS I 2007; Bron et al., 2017).

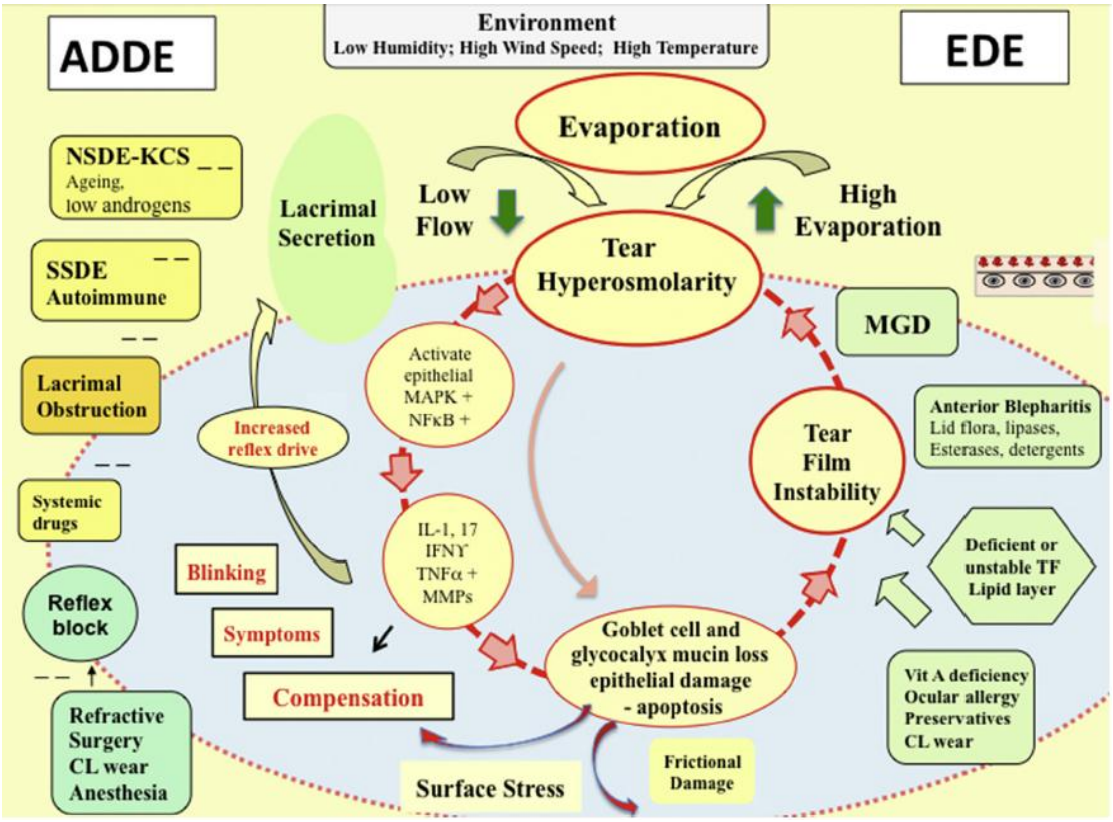


Figure 1.2 Vicious circle theory of DED pathology. From the Pathophysiology report of TFOS DEWS II. Bron et al. (2017). *Ocul Surf* **15**(3): 438-510.

1.17.2 Aqueous-Deficient Dry Eye

Aqueous-deficient dry eye (ADDE) is due to evaporation from the tear film in the presence of a reduced lacrimal secretion. Initially, tear hyperosmolarity results from evaporation at a normal rate from the tear film of reduced volume. Later, with the secondary occurrence of early tear film break up, an evaporative component may be added to the disorder.

There are various causes of ADDE, the simplest of which is due to a dysregulation of the LFU, which blocks the sensory drive to the lacrimal gland – so-called reflex blockade. This can result in a reduction of both tear secretion and blink rate. Causes include ocular surface anaesthesia due to topical anaesthetics and trigeminal nerve damage from injury and surgery, including refractive surgery, and from clinical disorders such as neurotrophic keratitis (NK). The delivery of aqueous tears is also reduced by obstruction of the lacrimal gland ducts, which can occur in any form of cicatricial conjunctival disease, such as trachoma, erythema multiforme, graft-versus-host-disease and chemical burns. Drugs in systemic use, such as antihistamines, beta-blockers, antispasmodics, diuretics and some psychotropic drugs that cause a reduction in lacrimal secretion are also risk factors for DED (Bron et al., 2017). The most common cause of ADDE is inflammatory infiltration of the lacrimal gland, particularly by T-cells, encountered most severely in autoimmune disorders such as Sjögren syndrome dry eye (SSDE) and more frequently, but with lesser

severity overall, in age-related, non-Sjögren dry eye (NSDE). Inflammation causes both acinar and ductal epithelial cell dysfunction and/or destruction and a potentially reversible neurosecretory block. In SSDE, a receptor block may also be caused by circulating antibodies to the muscarinic, M3 receptor. Inflammation is favoured by low tissue androgen levels. Sjögren Syndrome (SjS) is an auto-immune disease that attacks secretory glands, such as the lacrimal and salivary glands and other organ systems. It is termed primary when no other, defined systemic disease is present and secondary, when it is part of an additional, auto-immune connective tissue disease e.g. rheumatoid arthritis or lupus erythematosus. Sjögren Syndrome is caused by a combination of genetic, viral, environmental and hormonal factors which, in SSDE lead to T-cell infiltration of the lacrimal glands, instigating acinar and ductular cell death and lacrimal hyposecretion (Coursey and de Paiva, 2014). Similar events, of lesser degree are responsible for NSDE.

1.17.3 Evaporative Dry Eye

Evaporative dry eye (EDE) occurs when there is excessive evaporation from the tear film in the presence of normal lacrimal function. In this situation there is an opportunity to compensate for tear hyperosmolarity by means of a reflex increase in lacrimal secretion (Pflugfelder, Solomon and Stern, 2000; Bron et al., 2009; Arita et al., 2015).

Two forms exist, lid-related, or ocular surface-related. The commonest form of lid-related (intrinsic) EDE is non-cicatricial meibomian gland dysfunction (MGD), in which the terminal ducts of the meibomian glands are obstructed by hyperkeratinisation (Knop et al., 2011). Here, tear hyperosmolarity results from a quantitative and/or qualitative deficiency of the tear film lipid layer. It has been suggested that MGD-EDE is the commonest form of DED (Lemp et al., 2012; Korb and Blackie, 2015; Stapleton et al., 2017). Intrinsic EDE may also be the consequence of a prolonged blink interval, as in Parkinson's disease, or of poor lid/globe congruity, leading to defective tear film spreading. In ocular surface-related (extrinsic) EDE, tear film instability is initiated by conditions that impair ocular surface wettability, including short break up DED, xerophthalmia, ocular allergy, cicatrising conjunctivitis, topical preservative use and contact lens wear. In this case, it is early TBUT that initiates the tear film hyperosmolarity.

It will be noted that since tOsm can only rise in response to evaporative water loss, evaporation is the basis of tear hyperosmolarity in both ADDE and EDE and, in that sense, all forms of DED are evaporative; EDE is simply a hyper-evaporative state.

1.17.4 Hybrid forms of DED

There are various hybrid forms of DED, in which the salient features of both ADDE and EDE are present together and are summarised in the Pathophysiology section of the DEWS II report (Bron et al., 2017). This may be because the structural changes

responsible for each form occur together, as in SSDE, where lacrimal gland T-cell infiltration and obstructive MGD co-exist (Shimazaki et al., 1998), or in cicatricial conjunctivitis, where conjunctival scarring contributes both to ADDE and to a cicatricial form of MGD-EDE (Foulks and Bron, 2003; Radford et al., 2012). Additionally, the features of one form of DED may be added to that of another. Thus, it has been proposed that, in severe MGD-EDE, the compensatory reflex lacrimal response may be lost through the development of corneal insensitivity, and add a functional ADDE to the pre-existing EDE. Similarly, in ADDE, spreading of the TFLL can be impaired by severe aqueous tear deficiency, adding a functional EDE to the existing ADDE (Bron et al., 2009). A final and most important hybrid form of DED occurs in ADDE, where the onset of a shortened TBUT adds an evaporative element to the dry eye, which is of increasing severity the shorter the TBUT.

1.18 The vicious circle of DED

A sequence of events is described in dry eye, whereby tear hyperosmolarity, by damaging the ocular surface and destabilising the tear film, exacerbates the existing tear hyperosmolarity and completes a vicious circle of disease (Lemp et al., DEWS I 2007; Baudouin, 2007; Baudouin et al., 2013; 2016). This perpetuates and amplifies the DED, potentially dissociating the pathological events from their initiating cause. The sequence of events is as follows: there is evidence from a number of sources that tear hyperosmolarity stimulates a cascade of events in the ocular surface epithelium, involving stress- and mitogen-activated kinases (SAPK and MAPK) and NFκB signalling pathways (Li et al., 2004; Pflugfelder et al., 2005), and the generation and release of inflammatory cytokines (e.g. IL-1α, IL-1β, IL-8, IFNγ, TNF-α) (Lam et al., 2006; Pflugfelder et al., 2015), and proteases, such as matrix metalloproteinase-9 (MMP9) (De Paiva et al., 2006).

These activate and recruit inflammatory cells to the ocular surface which become an additional source of inflammatory mediators (Baudouin, 2001) and lead to a degradation and reduced expression of epithelial basal lamina, glycocalyx mucins, apoptotic death of surface epithelial cells (Yeh et al., 2003) and to loss of goblet cells. Goblet cell loss is a feature of every form of dry eye (Brignole et al., 2000; Kunert, Tisdale and Gipson, 2002), reflected by reduced tear levels of MUC5AC (Zhao et al., 2001; Argueso et al., 2002). Altered expression of glycocalyx mucins and breakdown of epithelial TJs, is a likely basis for increased ocular surface staining in dry eye and of the compromised ocular surface wetting that leads to early TBUT. This amplifies ocular surface hyperosmolarity, completes the vicious circle and establishes the mechanism that perpetuates the disease.

It has been emphasised by Baudouin et al., (2016) that the vicious circle offers *entry points* for any cause of DED (Baudouin et al., 2016); tear hyperosmolarity need not be the

starting point. Thus the chain of events leading to tear film instability may be initiated by several different disorders, such as ocular surface inflammation due to allergic eye disease, topical preservative toxicity, and loss of conjunctival goblet cells or altered mucin expression, due to xerophthalmia.

1.19 Prevalence and burden of DED

Stapleton et al., 2017 has recently summarised the prevalence of DED in the Epidemiology section of the TFOS DEWS II report, which based on symptoms, with or without signs, was between 5-50% (Stapleton et al., 2017). Studies based primarily on signs reported higher, although more variable rates, up to 75% in some populations. Asian ethnicity being a consistent risk factor, and prevalence was found to increase with age, with signs showing a greater increase per decade than symptoms. As in previous reports, women were more frequently affected than men using the Women's Health Study criteria (Uchino et al., 2011; Um et al., 2014), the difference, in this report, becoming significant with age. The report stressed the psychological and physical impact of pain and of disturbed vision on quality of life and its influence on work productivity, representing an important economic burden. The reported prevalence of DED varies markedly between studies on a global basis, varying geographically, and according to age and sex (Stapleton et al., 2017). Part of the difference in frequency may depend on the diagnostic definition of DED used, along with different techniques used, or in the characteristics of the study e.g. age, ethnicity, lifestyle and environmental factors.

As evidenced above, the incidence of DED is reported in vast numbers and is known to affect people worldwide, leading to symptoms of DED (irritation, itching, burning, foreign body sensation) being common reasons for seeking a medical opinion. This can represent a cause for increased health costs and reduced productivity at work that impacts on social and economic factors (Moss, Klein and Klein, 2000). DED can represent a significant financial burden either to the patients themselves or to the Health Service (Mertzanis et al., 2005) with expenses associated with medication and time needed to attend visits to an eye care professional. Furthermore, certain workplace environments have been shown to exacerbate symptoms of DED (Wolkoff et al., 2005) leading to reduced productivity (Uchino et al., 2014). The economic burden of DED in terms of cost of management has been calculated as $0.27 million in France to $1.10 million in the UK per 1000 patients managed by Ophthalmologists (Clegg et al., 2006). The total annual cost in the US was reported to be $3.84 billion (Yu, Asche and Fairchild, 2011) and in Singapore $0.15 million (Waduthantri et al., 2012).

1.20 Quality of life

Quality of life (QoL) is a standard of health, happiness and comfort of an individual that results in successful functioning in life. Several questionnaires have been used to assess the impact DED on the QoL, the most common of these being the utility assessment, a formal method for quantifying patient preferences for health outcomes (trading years of life for disease-free years) and the National Eye Institute Visual Function Questionnaire (NEI-VFQ). The latter questionnaire is a more general vision-related survey related to the subject's perception of their visual function and performance in relation to everyday tasks such as driving, watching television and using a computer. Evidence suggests that DED has a significant impact on the QoL of a sufferer, by causing pain, irritation, affecting general health and well-being, and reducing visual function and performance (Miljanović et al., 2007; Paulsen et al., 2014; Na et al., 2015). The ocular pain and decreased visual function experienced by DED sufferers has been stated to negatively affect function and daily activities, eventually leading to a correlation with anxiety and depression (Li et al., 2011; Le et al., 2012). Utility assessments suggest that severe DED has been equated with angina and mild DED with chronic psoriasis, with patients stating that if they were to live 10 more years they would give up on average 1.6 years of that time to be rid of severe DED (Schiffman et al., 2003).

1.21 Aims of the research

This background forms the basis of the research presented in this study, centred around the workings of the LFU. The following areas are addressed in this thesis:

- Development of a novel way to improve the utility of the Schirmer test,
- A study of the effect of evaporative suppression on tear osmolarity,
- A study to explore whether sensory drive from the surface of one eye influences the lacrimal responses of the fellow eye, and lastly
- To explore the influence of desiccating stress on tear osmolarity in the absence of, or in the presence of ocular surface anaesthesia.

Details of the methods used in various studies are summarised in Chapter 2.

Chapter 3 describes a method to standardise the Schirmer test by eliminating a major source of variation. Previous work by Holly et al., (1984) demonstrated a positive correlation between wetting length and RH i.e. lower RH resulted in reduced wetting. This is an important observation since the Schirmer test is regularly conducted in non-

standardised environmental conditions and outcomes could therefore underestimate the volume of tears actually produced in an individual. The Schirmer test was modified using a novel design to produce a water-impermeable sheath along the lines proposed originally by Holly et al., (1984) to prevent evaporation during the test. For the first time these experiments were conducted in standardised environmental conditions within the CEC, over a range of different RHs.

A positive correlation between pOsm and tOsm has been previously reported, with tOsm shown to increase with systemic dehydration (Fortes et al. 2011; Walsh et al., 2011; Ungaro et al., 2015; Holland et al., 2017). This technique could have utility in detecting dehydration in the elderly. However, the current method of sampling tears from the lower meniscus in the open eye could lead to a high level of false positives since evaporative effects could influence the sampled tOsm and DED, characterised by tear hyperosmolarity, is more prevalent with increasing age. In Chapter 4 two conditions of evaporative suppression (eye closure and high relative humidity) were adopted to circumvent this problem, as an innovative way to drive down tOsm to a stable, basal level that could be used more reliably to reflect pOsm in a clinical setting. Additionally, it is proposed that this basal value could be used as a personal baseline of tOsm against which to gauge the severity of hyperosmolarity in a patient with DED.

Lacrimal gland secretion is stimulated reflexly at a number of sites including the cornea and nasal mucosa. Current research has shown that a level of cross-connectivity exists in humans from the nasal mucosa (Heigle and Pflugfelder 1996; Gupta et al., 1997) and there is also evidence of corneal cross-connectivity in animals (van der Werf et al., 1996; Clarke and Bowsher, 1962; Pfaller and Arvidsson, 1988; Jacquin, Chiaia and Rhoadest, 1990); however, there is a lack of research on cross-connectivity in humans. An approach to this question was undertaken in Chapter 5, where the modified Schirmer test was used to explore the central connections of the LFU, using an indirect approach, comparing the relative effects of unilateral and bilateral sensory blockade, on the tear response. This is important in understanding of the effect that unilateral trigeminal nerve damage, such as that which occurs in neurotrophic keratitis can have on contralateral tear production. Can the healthy, fellow eye offset the loss of sensory drive on the affected side?

Several studies have demonstrated corneal staining and increased discomfort following exposure to desiccating stress with an unexpected absence in tear hyperosmolarity (Abushara and Pearce, 2013; Teson et al., 2013; Lopez-Miguel et al., 2014). This paradoxical finding is likely to be due to the presence of a normally functioning LFU in exposed study subjects, which maintains osmolar tear homeostasis, at least in the tear meniscus. In Chapter 6 the effect of desiccating stress on tOsm in both normal eyes and in patients with DED was investigated with a new methodology. Tear osmolarity was

measured with and without the influence of topical anaesthetics. This was used to block the afferent limb of the LFU and reduce compensatory, reflex tear secretion in an attempt to demonstrate the effects in the absence of a functioning homeostatic mechanism. This is relevant to DED patients in whom the LFU will already be compromised, to discover if any further capacity remains to protect the ocular surface.

All studies required experiments to be conducted under controlled environmental conditions of temperature, relative humidity and airflow and for this reason they were carried out in a controlled environment chamber (CEC).

Chapter 2:

METHODOLOGY

This chapter describes the methods and materials employed in the research reported here. Experiments were carried out at the Vision and Eye Research Unit (VERU), Anglia Ruskin University, Cambridge, UK. Additional details are provided, where necessary, in the relevant chapters.

2.1 Ethical approval

All studies were performed according to the Declaration of Helsinki. Ethical approval was obtained from the NHS Ethics Committee: South East Coast-Brighton and Sussex, and the University Ethics Committee. All subjects were informed about the nature of the study and gave written informed consent to their participation (Appendix II). They also received a letter about the study to take to their GP (Appendix III).

2.2 Participants for all studies

Participants were categorised into normal subjects and dry eye disease (DED) subjects, based on their responses to a symptom questionnaire (Appendix IV) and clinical assessment. Normal subjects were individuals with a normal ocular surface by history and by examination, according to defined criteria, recruited chiefly from students and staff at Anglia Ruskin University. DED patients were recruited from the cornea clinics of Mr Madhavan S. Rajan at Addenbrooke's Hospital and from the Cambridge group of the British Sjögren's Syndrome Association (BSSA). All participants were paid a small fee for their travel or time. Subjects of either sex, between the ages of 18 – 80 years, were recruited, according to the following criteria.

2.3 Inclusion criteria

2.3.1 Normal subjects

To join the study, a normal subject had to:

1. Be at least 18 years old and have full legal capacity to volunteer;
2. Have read and signed the IRB Informed Consent Document;
3. Be willing and able to follow participant instructions;
4. Have clear corneas and corneal staining <grade 2 on the Oxford scale;
5. Have a best corrected visual acuity of 6/6 or better in both eyes;

6. If a soft contact lens wearer, not to use their lenses for at least 8 hours before the procedure.

2.3.2 DED patients

DED patients who joined the study had to:

1. Be at least 18 years old and have full legal capacity to volunteer;
2. Have read and signed the IRB Informed Consent Document;
3. Be willing and able to follow participant instructions;
4. Have clear corneas other than the presence of punctate corneal staining ≥ grade 2 on the Oxford scale;
5. Have a best corrected visual acuity of 6/6 or better in both eyes;
6. If a soft contact lens wearer, not to use their lenses for at least 8 hours before the procedure.
7. Fulfill the internationally accepted definition of DED (Lemp et al., DEWS I 2007) and where relevant, the definitions of ADDE and/or EDE.

2.4 Exclusion criteria

2.4.1 Normal subjects

A normal subject who joined the study did **not**:

1. Have any systemic disease affecting ocular health;
2. Use topical medications and if receiving systemic medication was on a stable dose for the duration of the study;
3. Have active ocular disease;
4. Have any clinically significant lid or conjunctival abnormalities, neovascularisation, corneal scars or corneal opacities;
5. Have limbal or bulbar injection or corneal staining that was clinically significant;
6. Have worn hard or rigid gas permeable contact lenses within the previous 2 months;
7. Have had eye surgery or an eye injury within the previous 6 months.

2.4.2 DED patients

A DED patient who joined the study did **not**:

1. Have any systemic disease (other than Sjögren syndrome) affecting ocular health;
2. Use any systemic or topical medications that could affect ocular health except for artificial tears. Medication for a systemic condition that was stable and likely to remain so for the duration of the study was acceptable;
3. Have an active ocular disease other than DED, including that associated with Sjögren's

syndrome (SjS);
4. Have any clinically significant lid or conjunctival abnormalities, neovascularisation, corneal scars or corneal opacities; Meibomian Gland Dysfunction (MGD), however, was not a basis for exclusion, and was graded;
5. Have clinically significant limbal or bulbar injection, or conjunctival or corneal staining, other than that due to dry eye;
6. Have worn hard or rigid gas permeable contact lenses within the previous 2 months;
7. Have had eye surgery or an eye injury within the previous 6 months.

2.5 Further Classification of Dry Eye Disease patients

To satisfy the diagnosis of DED, patients were required to have been diagnosed with the condition for at least six months, to have an Ocular Surface Disease Index (OSDI) (Schiffman et al., 2000) score of ≥20, a corneal staining grade of ≥2 using the Oxford grading scheme (Bron, Evans and Smith, 2003), and a tOsm reading of ≥308 mOsm/L using the TearLab® osmometer (Sullivan et al., 2010). A TBUT of <10 seconds was considered as a DED indicator, but was not a requirement for inclusion.

2.6 Apparatus

2.6.1 Controlled Environment Chamber

Environmental studies were carried out in a PSR 'B' Series, Weiss Gallenkamp, controlled environment chamber (CEC), in which temperature, relative humidity and wind speed can be controlled and monitored. In preliminary studies it was found that, following the entry of subjects into the chamber, a period of up to 10 minutes was required for the CEC conditions to be restored to their set values. Figure 2.1 shows an example of the time taken for the CEC to equilibrate after being switched on. In this example the CEC reached its set values of 20ºC and 10% relative humidity (RH) within 10 minutes. At time points of 65 and 70 minutes the door of the CEC was opened for 10 and 20 seconds respectively. This did not affect the environmental conditions of temperature, RH with re-equilibration occurring within minutes. Therefore, for all the experiments, a period of 10 minutes after entry was allowed for CEC equilibration, before commencing data collection.

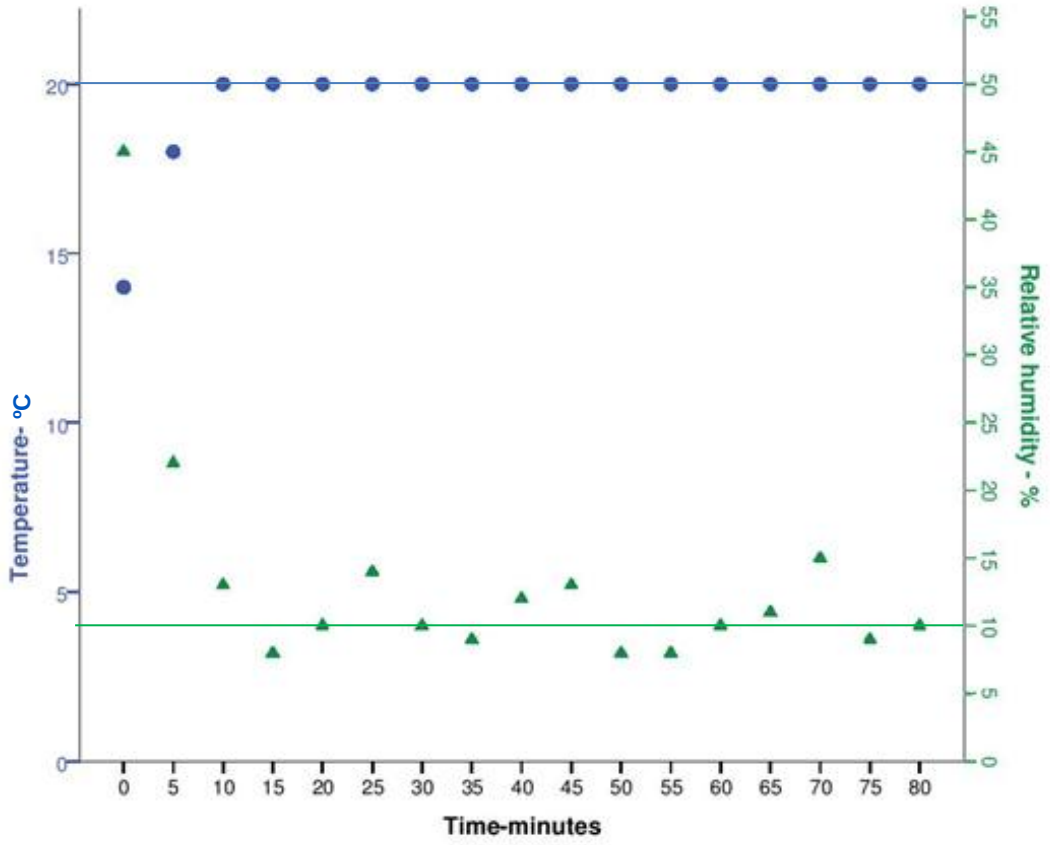


Figure 2.1 A scattergraph showing the timescale of equilibration of the CEC once turned on (at time 0), set to 20ºC and 10% RH. The CEC door was opened at time points of 65 and 70 minutes (Data collected 14th January 2014).

The CEC consists of a small chamber, with internal dimensions of 2.13m x 3.15m x 2.04m, in which two subjects and one examiner can be housed comfortably, together with additional, required investigational equipment (Figures 2.2-2.4). The air in the CEC circulates constantly with a laminar flow across the shorter width of the rectangular chamber, of 0.08 m/s. A unique feature of the CEC is an apparatus that produces a variable laminar flow, over a 0.6m square area in the range 0.15-1.1m/s, or over a 0.15m diameter area in the range 1.5-5.0m/s. This allows desiccating stress to be directed at the eyes and be modified for enhanced environmental conditions. Temperature can be altered between +5°C and +40°C with a fluctuation range of +/-2°C, and its relative humidity between 5% (at 10°C) and 70% (at 40°C) with a fluctuation range of +/-3%.

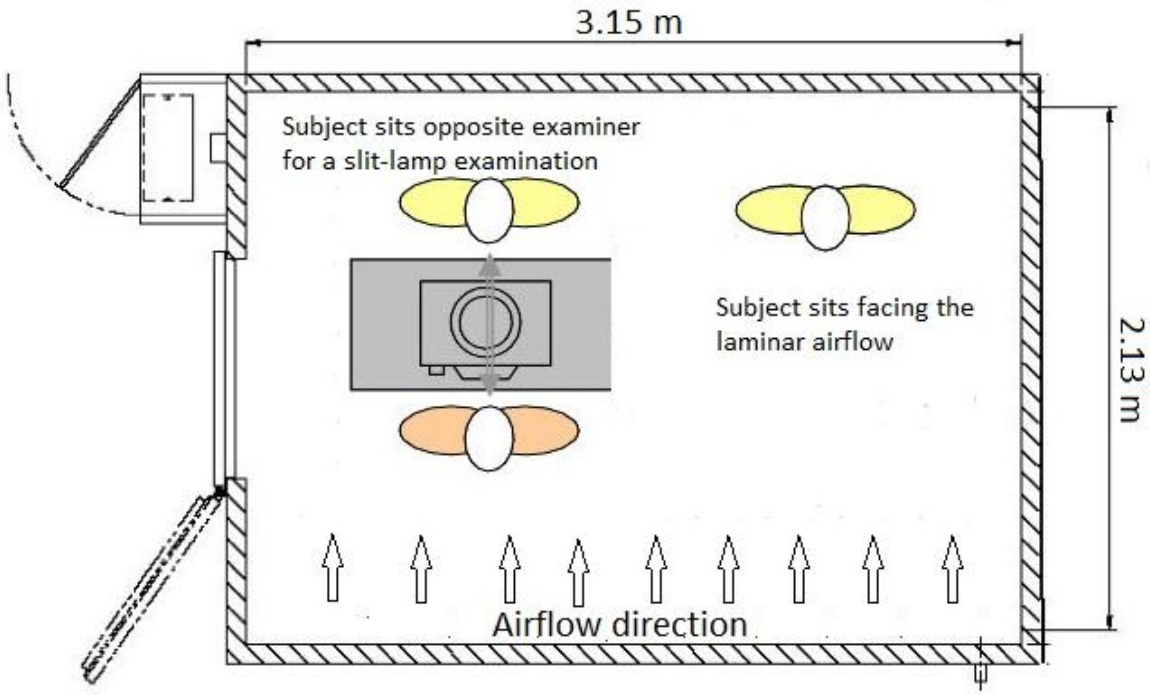


Figure 2.2 Dimensions and layout of the CEC.

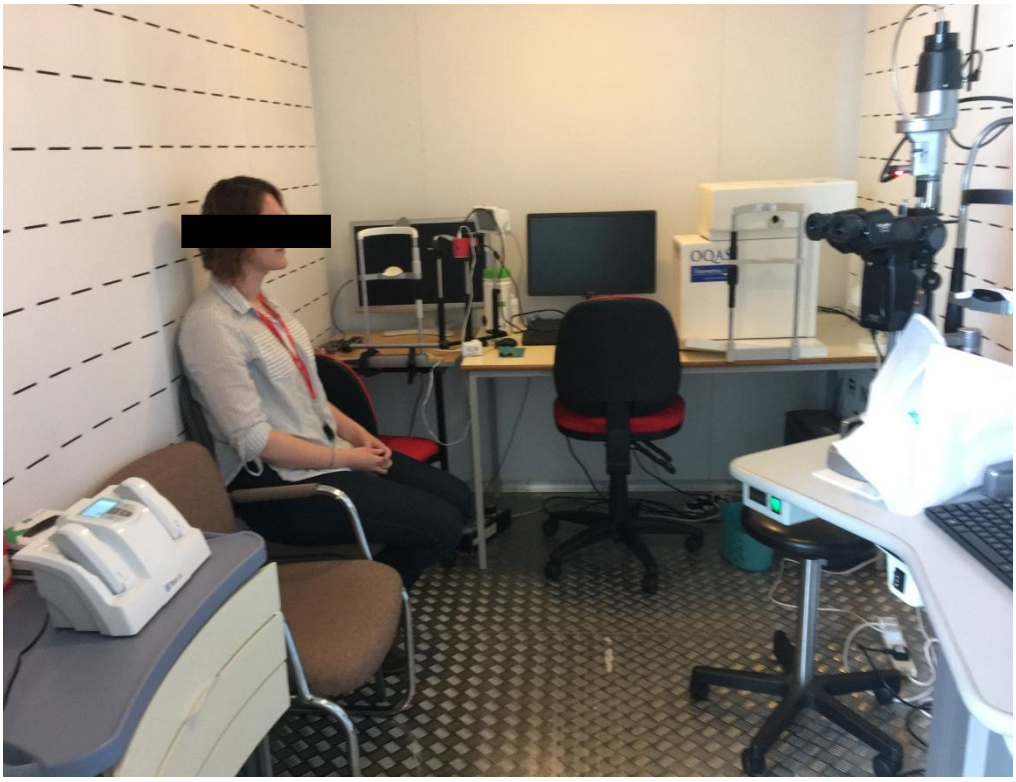


Figure 2.3 Subject sitting in the CEC facing the laminar airflow.

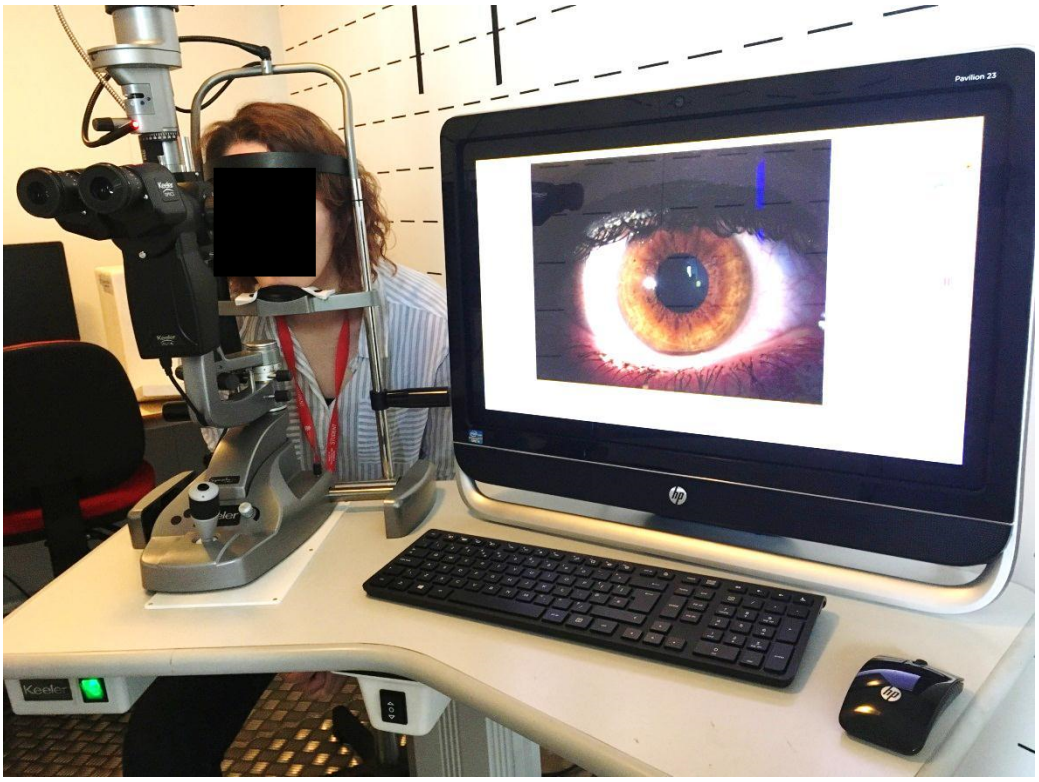


Figure 2.4 Subject sitting in the CEC for a slit-lamp examination.

In the planned experiments, 'standard workplace' conditions were set at a temperature of 23°C, a relative humidity of 45% and a laminar flow of 0.08 m/s (based on guideline in the Workplace Health Committee, OHS Information Sheet no. 5 1998). This latter flow rate is not negligible and the subject's placement in relation to the source of the laminar flow was standardised, with the subject facing the source (Figure 2.3). The RH was set at 5% to represent marked 'desiccating' conditions and 70% to represent a humid atmosphere with markedly 'evaporative suppression' conditions.

2.6.2 Slit-lamp examination

All slit-lamp examinations were performed using a Keeler Symphony Q 40H. This is a photo slit-lamp which was used for all clinical examinations of the anterior segment of the eye and to provide a permanent record of lid and ocular surface features, fluorescein TBUT and the ocular surface staining. At the end of any series of experiments the eyes were examined with and without fluorescein and any findings related to the experiments were recorded in the subject's record.

2.7 Clinical Assessments

At the time of recruitment all participants were classified as normal subjects or as dry eye patients on the basis of the above inclusion/exclusion criteria. All clinical history and data was documented on a recruitment record card (see Appendix VII). At recruitment,

assessments were carried out in the following order, to minimise interactions between tests. At other times, the order was determined by the specific protocol in use.

1) Clinical history
2) Visual Acuity
3) Ocular symptom questionnaire Index (OSDI)
4) Tear osmolarity
5) Slit-lamp examination with white light, followed by fluorescein instillation
6) Tear break-up time (TBUT) (with fluorescein)
7) Grading of ocular staining
8) Schirmer test
9) Meibography
10) Grading the quality of expressed meibum from the lower lid

2.7.1 Clinical History

Participants were interrogated according to a questionnaire (Appendix VII) regarding history of any eye or systemic conditions, including details of any medication and the duration of treatment, and also contact lens wear.

2.7.2 Visual Acuity

Visual acuity (VA) was measured using a Snellen chart at six metres (Pandit, 1994). VA was measured in the right and left eyes uncorrected and with current spectacles if worn.

2.7.3 Ocular Surface Disease Index (OSDI)

In this study the Ocular Surface Disease Index (OSDI), a validated questionnaire (Schiffman et al., 2000) with three domains, was used for the assessment of the symptoms, the influence of environmental factors and the functional impact of DED. There are 12 questions and participants are required to indicate, on a scale of 0-4, how frequently each symptom category is experienced. These choices are assigned numerical values, the total OSDI score is calculated on the basis of the formula OSDI = [(sum of scores for all questions answered) x 25] / (total number of questions answered) that are summed to create an overall score of symptom severity with a scale range of 0 to 100; in these studies a score for normal subjects was set at <20 (Schiffman et al., 2000). A mean OSDI score for mild to severe DED of between 3.9 and 13.4 has been reported in several other studies (Miller et al., 2010; Tian, Qu and Sun, 2016).

2.7.4 Tear Osmolarity

Tear hyperosmolarity as a pathogenic factor of DED was first hypothesised over 50 years ago (Balik, 1952). Since then several studies have demonstrated a positive correlation

between raised tOsm, a reduction in conjunctival goblet cell density and rose Bengal staining (Gilbard and Farris, 1979; Gilbard et al., 1988). Tear osmolarity has been proposed as a gold standard for DED diagnosis (Farris, 1994) and its measurement is recommended prior to refractive surgery, since a dysfunctional tear film may impair the surgical outcome (Versura and Campos, 2013). Historically, the earliest studies of tOsm used depression of freezing point and vapour pressure methods. Both techniques are dependent on the number of dissolved particles in the sample, 'colligative' properties that do not depend upon the size, weight or identity of the particles. In the freezing point method, tOsm is calculated from the depression of freezing point of the tear sample using the Clifton Nanolitre Osmometer (approximately 1 µL), which is directly proportional to the number of particles dissolved in the sample (Gilbard and Farris, 1979; Sullivan et al., 2005). Vapour pressure tOsm is calculated on the principle that, at the same temperature and pressure, the vapour pressure of a solution is depressed below that of the pure solvent, in proportion to the number of particles dissolved in the sample. Both these methods require time, proficiency at performing the test and are often subject to errors due to evaporation from the samples and inducing reflex tearing when sampling that can over or under estimate tOsm respectively (Terry and Hill, 1978; White, Benjamin and Hill, 1993). For the vapour pressure method, available sample volume (10µL) is a limiting factor, particularly in the study of aqueous tear deficiency (Tiffany, 2008).

Recently a new method for determining tOsm has been developed, based on measuring the electrical impedence of the tears. The TearLab® osmometer (TearLab Corp, San Diego, CA) is a microfluidic system which measures the electrical impedence of a 50nL sample taken up from the tear meniscus into a "lab on chip" test card, by capillary action (Figure 2.5) (Sullivan et al., 2005). This device (Figure 2.6) was used for all tOsm measurements made in the current studies. The device applies a calibration curve to the steady-state, temperature-corrected, electrical impedance of the tear fluid, to derive, indirectly (Pedersen-Bjergaard and Smidt 1952), a quantitative value of tOsm, the numerical value ranging from 275 to 400 mOsm/L. The osmometers used in this study were modified (in collaboration with the TearLab® company) to obtain values below the normal range of 270 mOsm/L, anticipated to be essential in Chapter 4. This involved attaching a serial cable to the port inside the device and connecting the TearLab® machine to a computer with a USB-to-serial converter. Establishing a HyperTerminal allowed the osmometer to communicate with the computer, and readings of low osmolarity numbers which ordinarily trigger a "Below Range" error on the device can then be viewed as a numerical value on the computer. There is no risk of evaporative loss during the sampling procedure using this machine, and since sampling can be completed within a few seconds the opportunity to induce reflex tearing is limited, although it is usual for the subject to experience a light sensation when the chip tip touches the lid margin. The

TearLab® is an instrument that provides rapid and reliable results for 'office use' rather than previously available equipment that was restricted to a research setting. The machine's most useful role being cited is as an objective diagnostic tool with the potential to detect DED in the early stages and to monitor the efficacy of therapy (Versura and Campos, 2013).

Procedure

Two TearLab® instruments were used in these experiments (one situated inside the CEC and one situated in the clinic), and calibration was verified before any experimental session. An electronic test card was used to run quality control measurements on both pens before any subject testing. A blue electronic test card was attached to a pen; a green light on top of the pen illuminates and the pen beeps when successfully attached. After approximately five seconds, the pen beeps again or the green light turns off. After the green light turned off, the pen was docked into the reader. The LCD displayed a code number. It was not necessary to enter a specific code for electronic check cards. To accept the code 'OK' was pressed. The LCD displayed a test result that must be between 331-337 mOsms/L to be acceptable. The same process was repeated with the other pen and notes of the date and the electronic check card test results recorded in a quality log. A TearLab® osmolarity control solution was then tested using each pen to verify the quantitative performance of the test card. This was carried out before every subject measurement on both instruments. A test card was attached to each pen, the pen would beep and the green light illuminate when the card was attached properly. The green light remained on until the control solution was collected. A sleeve was used to snap off the top of an ampoule which, was then turned upside down and the tip of the pen touched to the control solution. Once the pen had beeped again and the green light turned off, the pen was then returned to the reader within 40 seconds of collecting the sample and the code entered that matched the test card. After pressing 'OK' the LCD displayed the results; for the high control solution this must be between 330-346 mOsms/L for a pass. The same process was repeated with the other pen and notes of the date and the control solution test results recorded in a quality log.

To collect the tear sample the subject was instructed to direct the eye obliquely upwards and nasally, then a sample of tears was collected from the lateral tear meniscus by capillary action (Figure 2.7), into a disposable test chip (referred to as the 'card' by the manufacturers) mounted on a pen. The pen gave an audible and visible signal when sampling was complete and the impedance data collected. The pen was then docked into a reader, which calculated and displayed the osmolarity result. Sampling took about 1 second with little time for stimulating reflex tear secretion. In general, the subject experiences a slight tickle as the instrument tip comes into contact with the tear meniscus

and elements of the lid margin. In this study the subject was asked to grade the sensation at the time of sampling on a scale of 1-3, representing a sense of light touch or tickle (1), mild discomfort (2) to moderate discomfort (3). Readings of 3 were usually associated with difficulty in sampling and an increased risk of reflex tearing, and were discarded and not re-tested as a precaution. Some investigators have employed an approach in their studies that involved the subjects squeezing the eyes shut three times to 'create fresh tear fluid release from the lacrimal gland' before sampling tears from the meniscus (Fortes et al., 2011; Ungaro et al., 2015; Holland et al., 2017), but this was specifically avoided in present studies since it was important to record the osmolarity value of the unmodified meniscus tears.

One limitation of this conductivity system is that it is dependent on the number of charged particles in a solution, rather than the total number of particles present and therefore does not register the full osmolarity of the solution. However, for the tears, since ions make the major contribution to osmolarity with a very small contribution from charged proteins, this accounts for most of the particles responsible for tOsm. Tear urea, which would not be registered, would amount to about 6 mOsm/L and tear glucose, in a non-diabetic subject, for about 0.2 mOsm/L, so that the discrepancy is small. Importantly, several studies have found that the tOsm measurements made with the TearLab® device are highly correlated with results from the freezing point technique using a Clifton osmometer (Tomlinson *et al.* 2010) and with those from the Wescor 5520 Vapour Pressure Osmometer (Rocha et al., 2017).

The utility of the TearLab® osmometer used in this thesis is discussed further in Appendix XIII.

Figure 2.5 TearLab® disposable test card, displaying channel that collects tear meniscus sample.

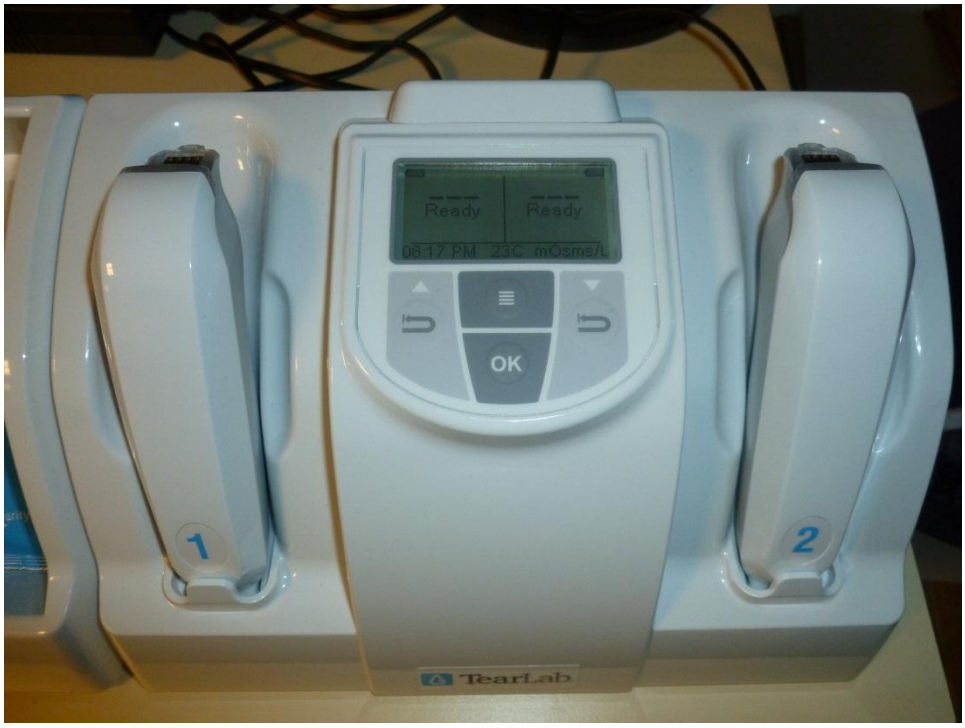


Figure 2.6 Tearlab® pens and docking station.

Figure 2.7 Sampling of the tears from the meniscus with the TearLab® osmometer.

2.7.5 Fluorescein instillation

Fluorescein instillation was used for the measurement of TBUT and for the detection of ocular surface staining at the end of clinical studies. A drop of single-dose sterile saline (Bausch and Lomb minims®) was applied to the Biotech® Fluoro, fluorescein ophthalmic strips U.S.P., each strip impregnated with 1.0mg of fluorescein sodium. As soon as the saline had saturated the impregnated portion, the excess was shaken off vigorously, with a single shake, into a receptacle and, with the lower lid pulled downwards the wetted tip was then gently tapped against the lower tarsal conjunctiva. This was carried out first on the right eye, to perform the TBUT and staining assessments, and then on the left eye.

In this study staining was graded using the Oxford grading scale, which has a grade range from 0-15, represented by punctate dots (cornea 0-5: and 0-5 on each of the nasal and temporal segments of the exposed conjunctiva) (Appendix V). For assessment of the cornea the upper lid was raised slightly to expose the whole cornea, with the eye in the primary position. Measurement of the nasal conjunctiva was carried out with the eye looking temporally and of the temporal conjunctiva with the eye looking nasally. A grade of ≥ 2 or over has been used as a diagnostic cut-off for DED (Bron, Evans and Smith, 2003; Bron et al. 2003; Bron et al., DEWS I 2007).

2.7.6 Tear Break-Up Time

The tear break-up time (TBUT) was performed after the instillation of fluorescein, using the slit-lamp at x16 magnification, with a blue light source and yellow absorbance filter in place. The subject was instructed to blink several times in order to mix the fluorescein with the tears and to create a homogeneously-stained tear film. Following this, the subject

was asked to blink three times and then instructed to stop blinking, at which point a stop-watch was activated and the time between the last blink and the onset of tear break-up was recorded. This was indicated by the appearance of an expanding dark zone or zones, within the stained film. At the first signs of the TBUT, the timer was stopped. If a blink occurred before the break-up of the tear film, the test was repeated. Three readings were taken and the average was used as the TBUT value for that eye. A value of <10 seconds was the cut-off for dry eye diagnosis. The TBUT and staining measurements were first performed on the right eye, then the left eye.

2.7.7 The Schirmer Test

The Schirmer test is a measure of tear production based on the wetting of an absorbent filter paper strip that is inserted over the lower lid margin at its outer third. Full reviews of Schirmer testing, including its controversial aspects, are given by Cho and Yap and Senchyna and Wax (Cho and Yap, 1993; Senchyna and Wax, 2008).

The Schirmer test Ia, without anaesthesia, is a measure of the reflex response of the lacrimal gland to ocular surface stimulation, acting via the lacrimal functional unit (LFU), the reflex system that regulates secretion on a moment to moment basis. In most cases, where the sensory and motor elements of the reflex system are intact, it measures the responsiveness of the lacrimal gland. Therefore, where glandular function is compromised by disease, as in ADDE (Lemp et al., DEWS I 2007), the responsiveness of the gland will be decreased and the Schirmer test result reduced. It is important to realise that, unlike fluorophotometry, the Schirmer Ia test does not assess the function of the lacrimal gland just prior to initiating the test but rather, the gland's reflex response to sensory stimulation of the ocular surface, during the test. It therefore depends not only on the health and function of the lacrimal gland but on the integrity of the LFU.

The Schirmer Ib test is performed in a similar manner, but after inducing topical anaesthesia in each eye. Since the instillation of a topical anaesthetic induces reflex tearing and the instilled volume itself could contribute to wetting of the Schirmer strip, time is allowed to elapse between drop instillation and performance of the test and in many accounts, excess fluid is removed from the conjunctival sac with an absorbent tissue before the test is carried out (Lamberts, Foster and Perry, 1979; Bawazeer and Hodge, 2003). Bilateral topical anaesthesia reduces lacrimal secretion by blocking the sensory limb of the LFU and removing sensory drive from the ocular surface to the gland (Li, Deng and He, 2012; Jordan and Baum, 1980). For this reason the Schirmer Ib value has been regarded as a 'basal' value by some authors (Jones, 1966) although this is denied by others (Jordan and Baum, 1980; Clinch et al., 1983; Afonso et al., 1999), because there are other inputs to secretion. It is better to think of it more specifically as the level of tear

production, which results when the combined input from each ocular surface is withdrawn, under defined experimental conditions.

The Schirmer II test is performed in a similar manner to the Schirmer Ib test, after inducing topical anaesthesia of the eye, but it is accompanied by stimulation of the nasal mucosa with a cotton swab. Like the Schirmer Ia test, assuming that the sensory and motor elements of the reflex system are intact, the Schirmer II test assesses the reflex response of the lacrimal gland, in this case in the absence of a sensory input from the ocular surface but with a strong sensory input from the nasal mucosa (Tsubota, 1998).

In response to some of the criticisms levelled at the Schirmer test, the Phenol Red Thread test (PRT) was designed: this is a cotton thread impregnated with phenol red. A colour change from yellow to red is elicited as the tears wet the thread which is pH sensitive. The PRT requires a much shorter measurement time (15 seconds compared to 5 minutes for the Schirmer) and is reported to stimulate a significantly reduced amount of reflex tearing and therefore represent tear flow rate or basal production (Hamano et al., 1983). However reports have suggested that the PRT is in fact measuring the uptake of tears residing in the fornical compartment along with the absorption characteristics of the cotton thread (Tomlinson, Blades and Pearce, 2001) and as such is no longer readily available for use in practice, and has been withdrawn from the Japanese diagnostic criteria for DED (Uchino et al., 2012).

Procedure

The Schirmer test: Biotech® Schirmer strips, Whatman filter paper number 41 were used in all experiments. The Schirmer strip was folded at the first mark (Figure 2.8), and the folded tip placed over the lower lid margin at its outer third. For purposes of standardisation, the strip was put in place first on the right eye and then the left, noting the precise time interval between the two. The subject was instructed to keep the eyes closed during the test; this is in accordance with European standard practice for the Schirmer test and also adopted in the USA. The strip was removed at precisely five minutes, first on the right and then on the left, and the wetted length recorded by marking the leading edge of the wetted front with a black ballpoint and measuring from the fold. If the strip was fully wetted before five minutes, the time to full wetting was recorded. The Schirmer strip was retained in the subject's file as a permanent record.

Figure 2.8 Unsheathed Biotech® Schirmer strip, with arrow showing where the strip is folded before insertion.

The Modified Schirmer Test: Chapter 3 outlines the design of a water-proof sheath used to modify the Schirmer test in an attempt to remove the influence of ambient humidity and airflow on the wetting results.

2.7.8 Meibography

Assessment of meibomian gland drop-out and expressibility is employed in the diagnosis of meibomian gland dysfunction (MGD) and is relevant to the presence of EDE, a sub-category of DED. A thickening of the meibum or blockage of the glands is present in the early stages of the disease and is demonstrated by simply applying pressure to the inferior lid margin and assessing the ease which the meibum is expressed and the quality of the meibum. As the condition progresses there can be a loss of meibomian glands due to increasing gland obstruction that may be irreversible (Bron and Tiffany, 2004). Drop-out of meibomian glands can be viewed using a Meibography pen (Arita et al., 2013). Meibography is a non-invasive technique used to assess the integrity of the meibomian glands. In the present studies, a hand-held infra-red meibography device was used (Japan Focus Co., Ltd) (Arita et al., 2013; Arita, 2013), in which intact glandular tissue appears pale against a dark background. With this method, images of the glands over the full extent of each lid, can be acquired and digitised for later analysis. For this research, gland loss or 'drop out' from the lower lid was graded, using the Arita schema (Appendix VI and Table 2.1). This pen-shaped meibography system utilises an LED illumination source (wavelength: 940 nm, TLN119 [F], Toshiba, Tokyo, Japan) and a highly sensitive CMOS video camera (250,000 pixels - Scalar Corporation, Tokyo, Japan) for image acquisition. It can be linked to a personal computer with a USB converter to provide a panoramic view of all the meibomian glands of the everted lower eyelids. The image was captured digitally with computer imaging software (EZCAP USB video grabber, Camsecure). Less than 1 minute was needed to observe the meibomian glands of both sets of lower eyelids.

Grade 0	Represents no loss of MGs (white area)
Grade 1	Represents a lost area (Black area) that is less than one-third of the total area
Grade 2	Represents a lost area between one-third and two-thirds of the total area
Grade 3	Represents a lost area more than two-thirds of the total area

Table 2.1 Non-invasive Meibography grading scale. From Arita et al. (2013). Cornea **32**(3): 242-247.

2.7.9 Meibomian gland expression

The meibomian secretion, liquid at body temperature, is a clear, yellowish oily fluid known as meibum. This clear liquid can be readily expressed from healthy meibomian orifices in young individuals. In the presence of MGD, the expressed material becomes increasingly viscous and cloudy (Wojtowicz, Butovich and McCulley, 2009). Grading of the quality of expressed meibum can be conducted in the upper and lower lids. In the present studies, meibum quality was graded following the application of firm pressure to the outer surface of the central third of the lower lid only (approximately eight glands) with a cotton bud. The quality of the expressed secretion was graded using the Foulks/Bron scheme on a scale of 0-3 (Foulks and Bron, 2003). The highest score from any of the expressed glands was taken as the overall score (Table 2.2).

Quality of expression
0 = clear 1 = cloudy 2 = granular 3 = solid N.B. zero expression with normal MG is not scored

Table 2.2 Grading of Meibomian expression. From Foulks and Bron (2003). *The ocul surf* **1**(3): 107-126.

2.7.10 Topical Anaesthesia

Topical anaesthesia was used in experiments conducted in Chapter 5 (Central Connectivity) and Chapter 6 (Sensory Blockade). In these studies it was necessary to achieve a prolonged period of anaesthesia of the ocular surface with a minimal degree of induced reflex tearing. Topical anaesthetics are used routinely in ophthalmology to conduct diagnostic tests. The two most commonly used are proxymetacaine 0.5% and tetracaine 0.5% and 1%, both available in single dose Minims®. Proxymetacaine is a benzoic acid ester, while tetracaine is a para-aminobenzoic acid ester, both working by blocking sodium conduction across nerve cell membranes, preventing rapid influx of sodium ions and depolarisation, and producing a reversible blockade of the sensory nerve endings. To improve solubility, commercial preparations are usually acidic, soluble hydrochlorides (proxymetacaine pH 4.64 and tetracaine pH 4.54) (Bartlett and Jaanus, 2008). Because of this acidity these agents sting on instillation, slightly less so in the case of proxymetacaine. Several studies using a visual pain scale have reported that

tetracaine is significantly more painful on instillation (Bartfield, Holmes and Raccio-Robak, 1994; Shafi and Koay, 1998). Shafi and Koay, (1998) reported that the stinging sensation lasted for 3.2 seconds with proxymetacaine and 22.1 seconds with tetracaine (Shafi and Koay, 1998).

The other key area of importance is the onset and duration of anaesthesia following instillation of these agents. Barfield et al., (1994) reported that both agents took effect approximately 30 seconds after instillation and that the duration of anaesthesia with proxymetacaine was 10.7 minutes, versus 9.4 minutes for tetracaine (Bartfield, Holmes and Raccio-Robak, 1994). In other reports, tetracaine has been reported to last in the order of 30 minutes (Lamberts, Foster and Perry, 1979; Lawrenson et al., 1998), and this was also confirmed in pilot studies (see Appendix XIII). Shafi and Koay, (1998) concluded that tetracaine was likely to be a more effective anaesthetic agent (Shafi and Koay, 1998). In general support of these findings, Weiss and Goren (Weiss and Goren, 1991), reported the mean duration of anaesthesia with proxymetacaine, to be 35 minutes while Polse et al., (1978) reported a value of 45 minutes (Polse, Keener and Jauregui, 1978), the latter group also showing that stronger concentrations of the drops increased the duration of effect.

A procedure was adopted in the current studies, to achieve the minimum of reflex tearing and the maximum level of anaesthesia with the anaesthetics used. To minimise reflex tearing, a single drop of the short-acting anaesthetic 0.5% proxymetacaine (Minims®) (Jones, 1966) was instilled first; this drop stings only slightly. The proxymetacaine drop was followed, 30 seconds later, by a drop of 1% tetracaine (Minims®). This drop would normally sting markedly when instilled into an unanaesthetised eye, but since the ocular surface has been rendered anaesthetic by the proxymetacaine, the instillation is not sensed by the subject and no additional reflex tearing is induced; it provides an increased density of anaesthesia (Adriani and Zepernick, 1964; Jordan and Baum, 1980). To maximise spreading and mixing of the drops and retention of the largest volume without overspill, the subject was asked to look up while each drop was instilled into the lower fornix and then asked to blink with the lower lid still drawn downwards. Finally, the eyes were closed to allow excess fluid to be drawn off and dabbed away at the outer canthus and along the closed lid margins. This sequence was followed by a period of up to 10 minutes of spontaneous blinking to allow for the drainage of any excess of tears, prior to any experimental procedure. The same general protocol was adopted for the instillation of saline drops, used as a control for topical anaesthetic drop instillation; in this case a drop of saline acted as a surrogate for each anaesthetic drop.

Chapter 3:

PILOT STUDY IN DEPTH AND DURATION OF TOPICAL ANAESTHESIA AND THE EFFECTS OF ENHANCED DESICCATING STRESS

Purpose: For two of the studies reported in this thesis, topical anaesthesia was used to investigate the responses of the lacrimal functional unit (LFU) to desiccating stress, in the absence of a sensory drive from the ocular surface (Chapters 5 and 6). In Chapter 5 a protocol of topical anaesthetic instillation was developed to provide a suitable depth and duration of ocular surface anaesthesia, with a minimal amount of reflex tearing. This involved the use of two different topical anaesthetics, commonly used in clinical practice. In Chapter 6 the effect of desiccating stress on the open eye, with and without sensory blockade was studied. Before commencing data collection, the level of desiccating stress that would be included in the protocol was piloted using the enhanced airflow system of the controlled environment chamber (CEC) to amplify the desiccating stress. These two areas were piloted as follows:

3.1 Pilot Study: Depth and duration of topical anaesthesia

Methods: Two normal male subjects, aged 70 and 79 years were seated in the clinic in uncontrolled environmental conditions. A single drop of 0.5% proxymetacaine (Minims®) was first instilled in the right eye and then in the left. This was followed, 30 seconds later, by 1 drop of 1% tetracaine (Minims®). The technique of instillation is described in full in the Methodology section (Chapter 2).

Corneal sensation was measured using a Cochet-Bonnet aesthesiometer (0.12 mm diameter filament) every five minutes for 45 minutes. The area measured was at a paracentral region of the cornea approximately 3mm from the limbus at 6 o'clock. Corneal contact and the defined force applied was registered by the smallest visible bending of the nylon monofilament. The filament length was reduced from its maximum (6 cm) in 1cm steps, until the subject indicated that the stimulus had been felt. 'Total anaesthesia' was recorded when the subject was unaware of a touch sensation following contact of the 1cm length filament. 'Recovery' was recorded when the subject was aware of a touch sensation with the 1cm filament.

Results: Depth of corneal anaesthesia for the two subjects is shown in Table A13. At baseline the corneal sensation of both subjects was recorded as 6cm. Total corneal anaesthesia was recorded in both subjects, at five minutes after instillation of the

anaesthetics, with recovery of corneal sensation being recorded, again in both subjects, at 45 minutes.

Time (minutes)	Subject 1		Subject 2	
	Length of filament (cm)			
	R	L	R	L
Baseline	6	6	6	6
Instillation of drops				
5	/	/	/	/
10	/	/	/	/
15	/	/	/	/
20	/	/	/	/
25	/	/	/	/
30	/	/	/	/
35	/	/	/	/
40	/	/	/	/
45	1	1	1	1

Table 3.1 Corneal sensitivity before anaesthesia and measured at five minute intervals for 45 minutes after anaesthesia. [/] = No response to touch with 1cm filament.

Discussion: In both subjects, total corneal anaesthesia was recorded at 5 minutes after the instillation of the anaesthetics, and recovery of corneal sensation by 45 minutes. This is in line with an experiment by Lawrenson (1998) who reported total anaesthesia after one minute of instilling either amethocaine (i.e. tetracaine), oxybuprocaine or proxymetacaine, with recovery of corneal sensation occurring at 30 minutes; and a return to baseline sensitivity by 45 minutes. The slightly extended period of time before recovery recorded in this study was attributed to the additive effect of using two anaesthetics in each eye. In response to the results here, the protocols planned for the main experiments followed the drop instillation routine outlined above. This minimised reflex tearing due to drop instillation, since the proxymetacaine drop stings only slightly, and tetracaine, that would normally sting markedly on instillation, is secondly instilled, into a previously anaesthetised eye. The combination drop regime was considered to provide an increased density of anaesthesia (Adriani and Zepernick 1964; Jordan and Baum 1980).

3.2 Pilot Study: Increased desiccating stress with sensory blockade

In this pilot study an approach was explored in which the level of desiccating stress to the eyes was amplified by exposing the subject to an enhanced airflow while the subject was seated in the CEC at an RH of 5% (completed on 22nd January 2014).

Methods: One normal male subject, aged 79 years was seated in the CEC set to 5% RH and 23ºC and with a laminar airflow of 0.08m/sec. Additionally the subject was exposed to an increased airflow directed to the head, with the subject facing the source (Chapter 2, Methodogy) which was activated 10 minutes after the anaesthetic instillation. The

anaesthetic instillation procedure (see above) was initiated shortly after entering the CEC, and the enhanced airflow was activated 10 minutes after entry. The subject faced the enhanced airflow system with both eyes open and blinking spontaneously.

The intention was to observe the eyes at 15 minute intervals, however at 5 minutes the subject volunteered a change in vision in the left eye and the experiment was concluded.

Results: The appearance of the two eyes before the experiment and following fluorescein instillation, are shown in Figure A13 a and b. They were considered to be free of significant corneal staining. The image of the right tear film is imperfect since it shows the imprint of the upper lid and associated meniscus induced thinning prior to lid elevation. Therefore a further image of the right cornea, demonstrating normality, acquired on another occasion, is included in Figure A13 c (obtained 11th February 2015). Figures A13 d and e show the appearance of the right and left corneas 5 minutes after initiating enhanced airflow, and following a further instillation of fluorescein. Each cornea showed extensive, confluent, punctate epithelial staining, occupying the exposed regions of the corneas. The subject has a relative ptosis, and the upper extent of the stained region corresponded to the position of the upper lid margin. The upper part of the cornea was protected by the upper lid.

Discussion: Instillation of anaesthetic eye drops and exposure of the eyes to 5% RH for 10 minutes followed by an additional exposure of the eyes to increased airflow of 2m/sec for a further five minutes, resulted in an diffuse and extensive punctate epithelial keratopathy (PEK) affecting the exposed cornea. The subject underwent a slit-lamp examination again the following day, at a different site, and the PEK had resolved. To gauge the compensatory levels of the LFU in conditions of desiccating stress, topical anaesthetics were used to block the afferent signals. It was apparent from this study that, in a situation of dense corneal anaesthesia, during exposure to a combined source of desiccating stress (5% RH plus an enhanced, directional airflow), the absence of an adequate compensatory tear response resulted in corneal damage. It cannot be excluded that some kind of toxicity due the topical anaesthetics might have interacted with the desiccating stress to cause this response. Because of the speed of onset and severity of the tissue response, it was decided that the use of an enhanced desiccation approach represented a significant risk to subjects, and therefore this approach was not adopted for studies in general. The protocol was therefore adapted to include a low RH (5%) with a laminar airflow of 0.08 m/sec as used in the other studies.

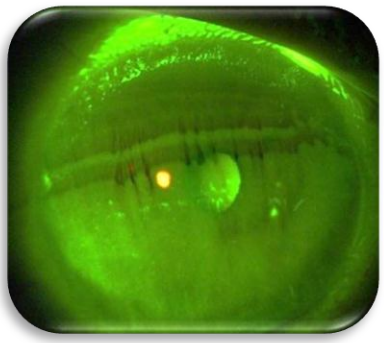
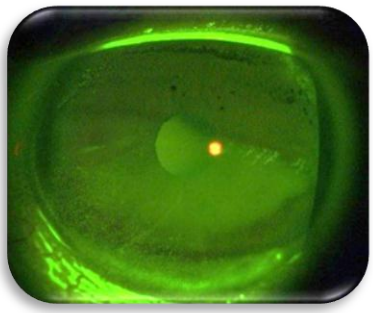
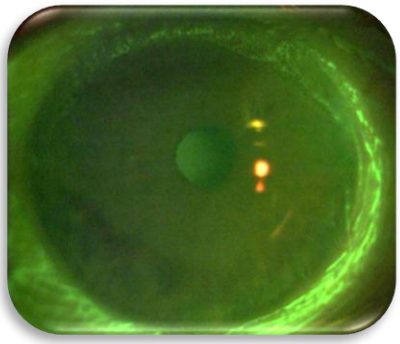
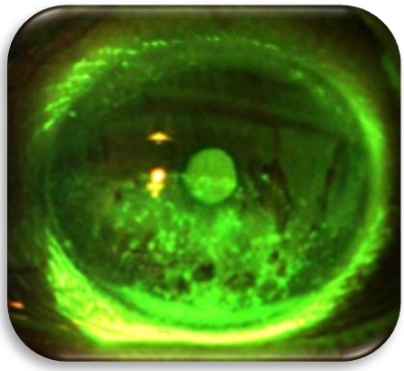
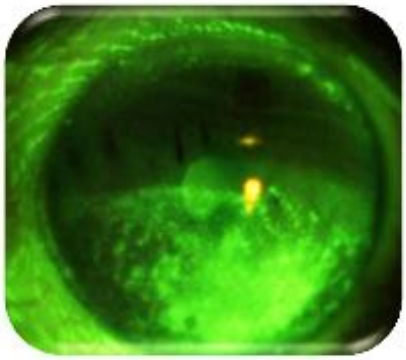
	Right eye	Left eye
Pre-exposure to desiccating stress	 a	 b
	 c	
Post-exposure to desiccating stress	 d	 e

Figure 3.1 Fluorescein staining images of the right and left eye following instillation of anaesthetics and pre and post-exposure to enhanced desiccating stress. The brightness and contrast of the images have been adjusted to highlight features of corneal staining.

Chapter 4:

STANDARDISING THE SCHIRMER TEST BY ENCLOSING THE STRIP IN A WATERPROOF SHEATH

These experiments were designed to study the effect of different relative humidity (RH) levels on the Schirmer test in closed eye conditions and to determine whether shielding the paper from evaporation, using a waterproof sheath would have an effect on wetting length in these conditions.

4.1 INTRODUCTION

Tear production can be measured accurately in standardised laboratory conditions by fluorophotometry, a dye-dilution technique in which a standard amount of fluorescein is instilled into the conjunctival sac and the fall in tear film dye concentration is measured photometrically over time (Tomlinson and Khanal, 2005). The purpose of this technique is to measure the steady-state level of tear production (predominantly lacrimal secretion) in controlled environmental conditions. Other dye-dilution techniques, of lesser accuracy, are also available (Macri, Rolando and Pflugfelder, 2000; Kaye et al., 2001). In non-specialised units or clinics, because of time constraints, cost and technical considerations, it is more usual to measure tear production by the Schirmer test. This test is performed bilaterally and nowadays conducted with the eyes closed, which reduces light exposure and evaporative water loss from the ocular surface during the test. According to how the test is performed, it has been stated that it can provide information about reflex or basal tear production. Despite its reported shortcomings, the test has regularly been included among the battery of assessments advocated for the diagnosis of dry eye disease (DED) (Balik, 1952; Wright and Meger, 1962; Lemp, 1995; Tomlinson et al., DEWS I 2007; Wolffson et al., 2017). The result provides information about the functionality of the lacrimal functional unit (LFU) and since it is generally assumed that the reflex neural pathways are intact, the Schirmer value is taken as a measure of the responsiveness of the lacrimal gland. A normal Schirmer value is taken as evidence of a healthy lacrimal gland and an adequate aqueous tear supply. The proportion of wetting of the Schirmer strip could be used to differentiate between phenotypes of DED; a reduced value being the usual diagnosis of aqueous-deficient dry eye (Bron et al., 2009).

A full review of Schirmer testing, including its controversial aspects, is given by Senchyna and Wax (Senchyna and Wax, 2008). In brief, the Schirmer test, without anaesthesia (referred to here as Ia), is a measure of the reflex response of the lacrimal gland to ocular surface stimulation, while the Schirmer test with topical anaesthesia in each eye (referred

to here as Ib) has been regarded as a 'basal' value by some authors (Jones, 1966), although this is denied by others (Clinch et al., 1983; Afonso et al., 1999), because there are other inputs to secretion. The Schirmer II test is performed after inducing topical anaesthesia of the eyes, but accompanied by stimulation of the nasal mucosa with a cotton swab. Like the Schirmer Ia test, assuming that the motor elements of the reflex system are intact, the Schirmer II test assesses the reflex response of the lacrimal gland to a strong sensory input from the nasal mucosa, in this case in the absence of a sensory input from the ocular surface.

Various authors emphasise the high variability and low reproducibility of the Schirmer test which undermines its value in the diagnosis of DED (Loran et al., 1987; Kallarackal et al., 2002; Serin et al., 2007; Sullivan et al., 2010), except, perhaps in patients with moderate to severe aqueous deficient dry eye where there is greater reliability (Nichols et al. 2004). Part of the variation is likely to be technical and part biological, reflecting the sensitivity of the reflex system to small environmental changes. Some portion of the tear production may be lost, by nasolacrimal drainage via the puncta (de Roeth, 1953; Norn, 1965).

A reason for variability of the test in differing environmental conditions was identified by Holly and colleagues (Holly, Lamberts and Esquivel, 1982; Beebe, Esquivel and Holly, 1988; Holly, 1994), who demonstrated that evaporative water loss from the Schirmer paper strip reduces the wetting length over the 5 minute course of the test, leading to an underestimation of reflex tear production. This could render the test sensitive to the ambient conditions of the test, with the expectation that the wetting length will be particularly reduced in conditions of decreased humidity or high and/or turbulent, airflow. Holly et al., (1982) stated that an increased draft in the vicinity of the Schirmer strip could increase the evaporation rate "several-fold" (Holly, Lamberts and Esquivel, 1982). These authors showed that this artefact could be prevented by enclosing the Schirmer strip in a water-impermeable sheath to prevent evaporation (Holly, Lamberts, and Esquivel, 1982). According to their findings, in the absence of protective sheathing, for a given, true tear flow rate, wetting length would be positively correlated with relative humidity; and the lower the relative humidity, (therefore the higher the evaporative loss from the strip), the lower the predicted wetting length.

In the studies by Holly and colleagues (1982; 1988; 1994), it was shown that the total evaporative loss from the wetted strip increased linearly with wetting length, due to the increased wetted area exposed (Holly, Lamberts, and Esquivel, 1982). Eventually this reached a steady state in which the uptake rate was equal to the total evaporation rate. It follows that in any given condition, the wetting length will be increased by cladding the paper strip in a water-impermeable sheath to prevent evaporation. In the reports mentioned above by Holly and colleagues (Holly, Lamberts and Esquivel, 1982; Beebe,

Esquivel and Holly, 1988; Holly, 1994), Black Ribbon No. 589 filter paper was used for their Schirmer experiments, with and without sheathing. The temperature and airflow levels used in the experiment were not disclosed, other than to state that it should be controlled, “avoiding unpredictable air turbulence around the strip”. In the experiments described below, the commercially available Biotech® Schirmer strips (Whatman No. 41) were used, Whatman No. 41 filter paper having been found to have a similar wetting curve to Black Ribbon No.589 (Figure 4.1) (Holly, Lamberts, and Esquivel, 1982), and the environmental conditions standardised using a controlled environment chamber (CEC).

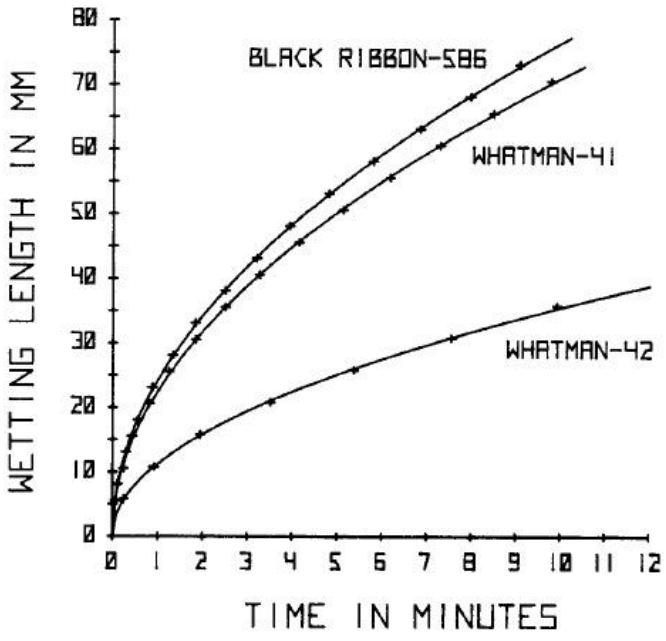


Figure 4.1 Wetting curves obtained for Black ribbon and Whatman 41 filter paper in free absorbtion, conducted in an environment where temperature and humidity could be controlled and airflow eliminated (conditions undisclosed). From Holly et al. (1982). *Curr Eye Res* **2**(1): 57-70).

The following experiments were designed to expand on those of Holly and colleagues and to investigate the quantitative effect of decreased humidity on Schirmer wetting length, in the presence or absence of a protective sheath. The outcome of these studies was also relevant to studies on central nervous connectivity (Chapter 6).

4.2 HYPOTHESIS

It was proposed that, for a given, true tear flow rate, cladding the Schirmer strip in an impermeable plastic sheath would significantly increase the Schirmer wetting length compared to the unclad condition in inverse proportion to the relative humidity.

4.3 MATERIALS AND METHODS

4.3.1 Subject enrolment

Eight normal subjects were recruited, three male and five female, aged 36 years ± 24.22 (mean ± SD) (see Chapter 2 for inclusion and exclusion criteria). A closed-eye, sheathed vs. unsheathed Schirmer test was performed bilaterally in all eight subjects, in RH conditions of 5%, 15%, 25% and 45%. Subject three also took part in the experiments featured in chapters five and six, and subject four also took part in the experiments featured in chapters four, five and six. At least one month was allowed to elapse before these subjects were invited to participate in the other experiments. The nature of the research was explained to the subjects and written informed consent was obtained at least 24 hours before commencement of the experiment. Table 4.1 contains the ocular profiles of the right eye of normal subjects recruited for this experiment.

Subject	Age (years)	Gender	tOsm (mOsm/L)	Corneal stain	Schirmer wetting (mm)	OSDI	TBUT (seconds)	Meibography
1	20	F	292	0	19	6.25	10.7	0
2	19	F	283	0	19	17.5	11.3	0
3	22	F	293	0	28	2.08	10.6	0
4	32	M	297	0	17	0	10.2	0
5	21	F	298	0	24	4.16	11.8	0
6	25	F	295	0	33	0	14.1	0
7	79	M	301	1	17	12.5	10.2	0
8	70	M	293	0	15	6.25	13.7	0
Mean ± SD								
	36 ± 24.2	5F:3M	294 ± 5.37	0.13 ± 0.35	21.5 ± 6.28	6.09 ± 6.16	11.58 ± 1.53	0 ± 0

Table 4.1 Profiles of the normal subjects consented for this study.

4.3.2 Equipment

Construction of the Schirmer sheath

Lightweight sheaths were constructed from plastic sheeting (Fellowes ImageLast) laminating pouch sheets. The harder outer layer is made of polyethylene terephthalate plastic and the softer inner layer is made of ethylene-vinyl acetate plastic. Briefly, each sheath was constructed in the following way: using a thin heating iron (BaByliss hair

straightener), the laminating sheets were sealed on three sides after 3 seconds of heat contact. The sheaths were then cut into 4cm by 2cm pockets, 80 microns thick, which permitted the insertion of a Biotech® Schirmer strip (Figure 4.2), an unbonded, porous paper strip 35mm by 5mm (Whatman No. 41), allowing 6 mm of the insertion end of the strip to project beyond the top edge of the plastic housing. In use, the top end of the strip was folded at the first mark on the Biotech® Schirmer strip at a right angle (Figure 4.3), in order to hang the strip over the lid margin at the time of placement. When the sheathed Schirmer strip was in place for measurement of reflex tearing, its presence was generally indistinguishable by the subject, from that of an unsheathed strip. The steps involved in inserting the Schirmer strip into the sheath are shown in Figure 4.4 A-F.

Figure 4.2 A Biotech® Schirmer strip, an unbonded, porous paper strip 35mm by 5mm (Whatman No. 41).

Figure 4.3 A Biotech® Schirmer strip, enclosed in a lightweight sheath constructed from plastic sheeting.

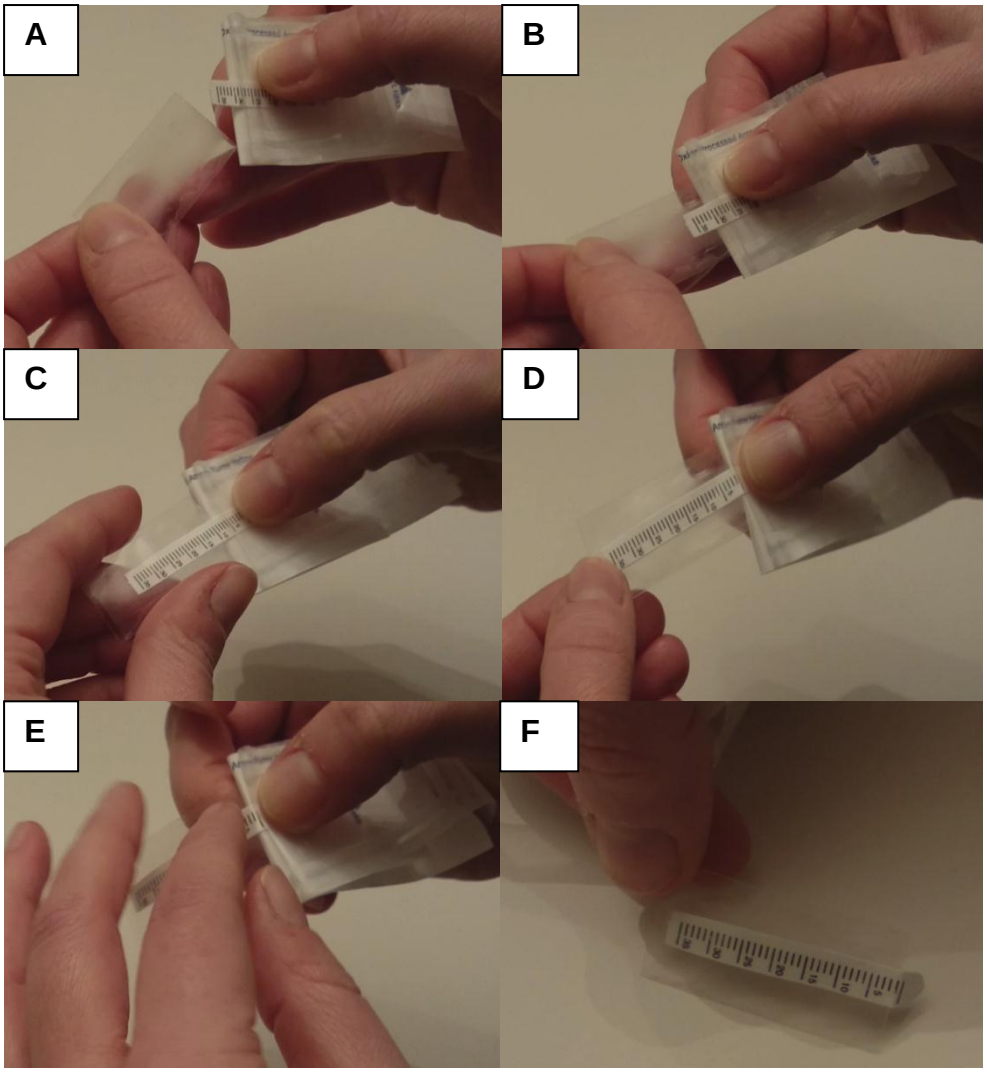


Figure 4.4 A-F Images of how the Schirmer strip was inserted into the sheath before use on a subject. This was completed approximately 10 minutes before use. New sheaths were created for each subject.

4.3.3 Protocol

The experiments were conducted in the Weiss-Gallenkamp controlled environment chamber (CEC). Tests were conducted over a range of RH in two sessions on different days, with the temperature set to 23°C and a laminar airflow of 0.08 m/s. In session one the Schirmer test was conducted at RH of 25%, 15% and 5%, with a 10 minute break between each test, to facilitate a change to the CEC RH setting and time for re-equilibration to the new RH level; this also provided a comfort break for the subject. Session two was conducted at a RH of 45% on a separate day. Eyes received either a standard Schirmer strip or a Schirmer strip clad in a waterproof sheath, on a random basis, decided by the toss of a coin.

For each set of studies after entering the CEC, subjects were seated with their eyes open looking straight ahead and blinking spontaneously for ten minutes while the conditions of the CEC stabilised. A closed-eye Schirmer test was then performed in both eyes as near simultaneously as possible, inserting the first strip in the right eye and the second in the

left eye with the eyes closed for 5 minutes immediately after insertion of both Schirmer strips. The time between the insertion of the first strip and the insertion of the second strip was measured and strips were removed for reading in the order of their insertion and at the same time interval, at the end of the 5 minute test. The wetting length was marked with a black ballpoint and recorded. If the wetting length of the Schirmer strip was oblique then the mid-point of the line was taken as the measured point. If full wetting occurred before this time, the time was noted. In subjects where the Schirmer wetting length reached 35mm before the end of the 5 minute (300 seconds) test period, an extrapolated value was used for analysis, as employed by Gupta et al., (1997), whereby the wetting length was divided by the number of seconds in which full wetting occurred and this value was then multiplied by 300 seconds (Gupta et al., 1997). Both Schirmer strips were retained in the subject's record. Movement of all individuals present in the CEC was kept to the necessary minimum during the studies, in order to avoid turbulent airflow and to maintain standardisation of the environmental conditions.

At the end of the series of experiments the eyes were examined with and without fluorescein and any findings were recorded in the subjects' records. Slit-lamp photographs, including fluorescein staining, were also taken; an example of a record card is shown in Appendix VIII. Figure 3.5 shows a timeline of 70 minutes over which the subjects were exposed to the three different RH.

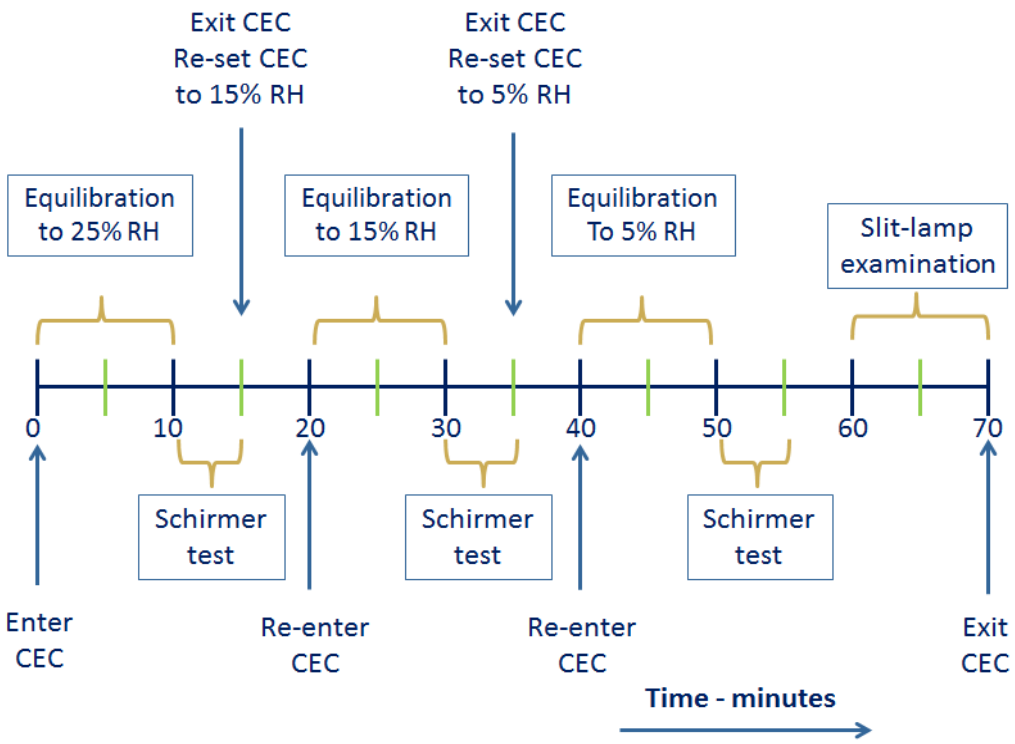


Figure 4.5 Timeline of visit.

4.3.4 Statistical analysis

A paired t-test was used to compare the wetting length of the sheathed and unsheathed Schirmer tests in the different conditions of RH. The mean difference in Schirmer wetting lengths between the sheathed and unsheathed values in the four different experiments were analysed using a repeated measures one way ANOVA. The Bonferroni method was used to make adjustments for multiple comparisons. The Shapiro-Wilk test was used to evaluate normality of distribution. Differences were considered statistically significant with *P* values less than 0.05. Data is presented as mean ± standard deviation. Analyses were performed using SPSS 20.

4.4 RESULTS

The results presented here show the comparison of the Schirmer wetting length between the two eyes in an individual, with one strip sheathed by an impermeable membrane and that in the fellow eye unsheathed.

5% Relative Humidity

The raw data of Schirmer wetting lengths for each participant at 5% RH can be found in Table 4.2. The mean Schirmer wetting length in the sheathed condition was 23.9 ± 14.9mm and in the unsheathed condition 11.9 ± 9.6mm (Figures 4.6 and 4.7). The mean difference in wetting length was 11.9 ± 8.6mm, representing a 49.3 % mean increase in Schirmer wetting length on the sheathed side, $t_{(7)} = 3.911$ ($p = 0.006$).

Participants (gender/age)	**1** F/20	**2** F/19	**3** F/22	**4** M/32	**5** F/21	**6** F/25	**7** M/79	**8** M/70
5% RH								
Sheathed side	L	L	R	R	L	L	R	R
Test time (seconds)	300	300	300	300	300	185	300	300
Sheathed wetting length (mm)	7	18	23	31	17	57*	21	17
Unsheathed wetting length (mm)	4	9	15	9	17	32*	5	4
Time corrected difference in wetting length (mm)	3	9	8	22	0	25*	16	13
Mean difference in wetting length ± SD (mm)	11.9 ± 8.6mm ($p = 0.006$)							
Difference in wetting length (%)	42.9	50.0	34.8	71.0	0	43.8	76.2	76.5
Mean difference in wetting length ± SD (%)	49.3% ± 25.8							

Table 4.2 Sheathed and unsheathed wetting length results for each participant at 5% RH.
* For statistical analysis, the extrapolated Schirmer value at 5 minutes was used for samples in which the strip was completely wet before the 5 minutes.

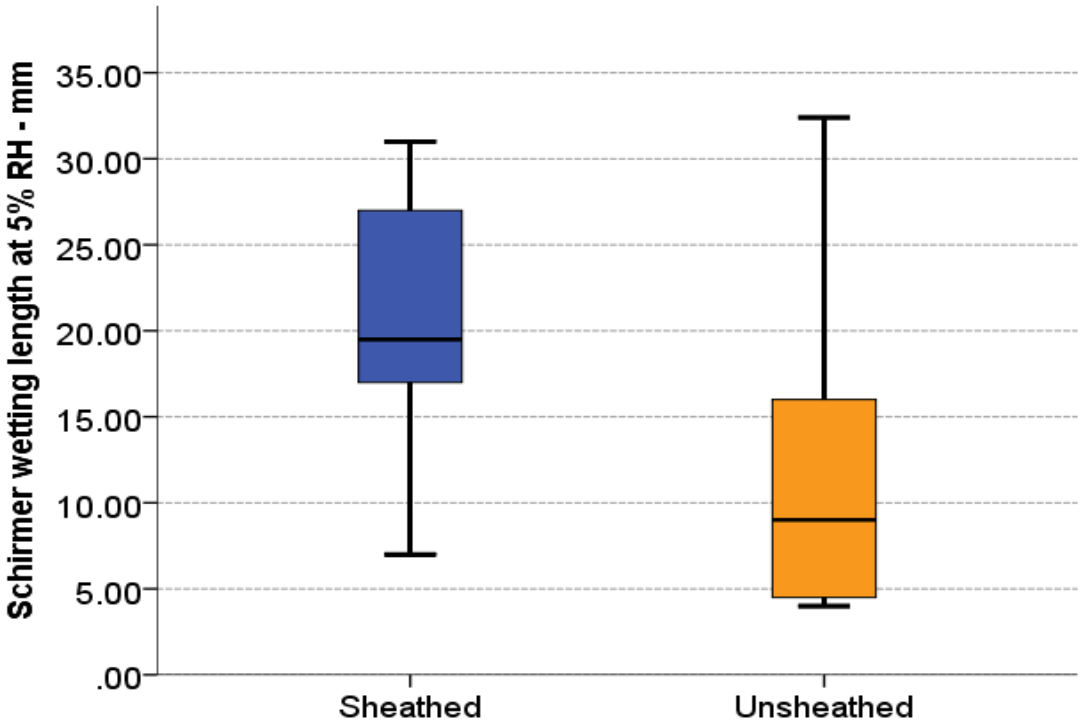


Figure 4.6 Boxplot showing the Schirmer wetting length in the sheathed and unsheathed conditions at 5% RH.

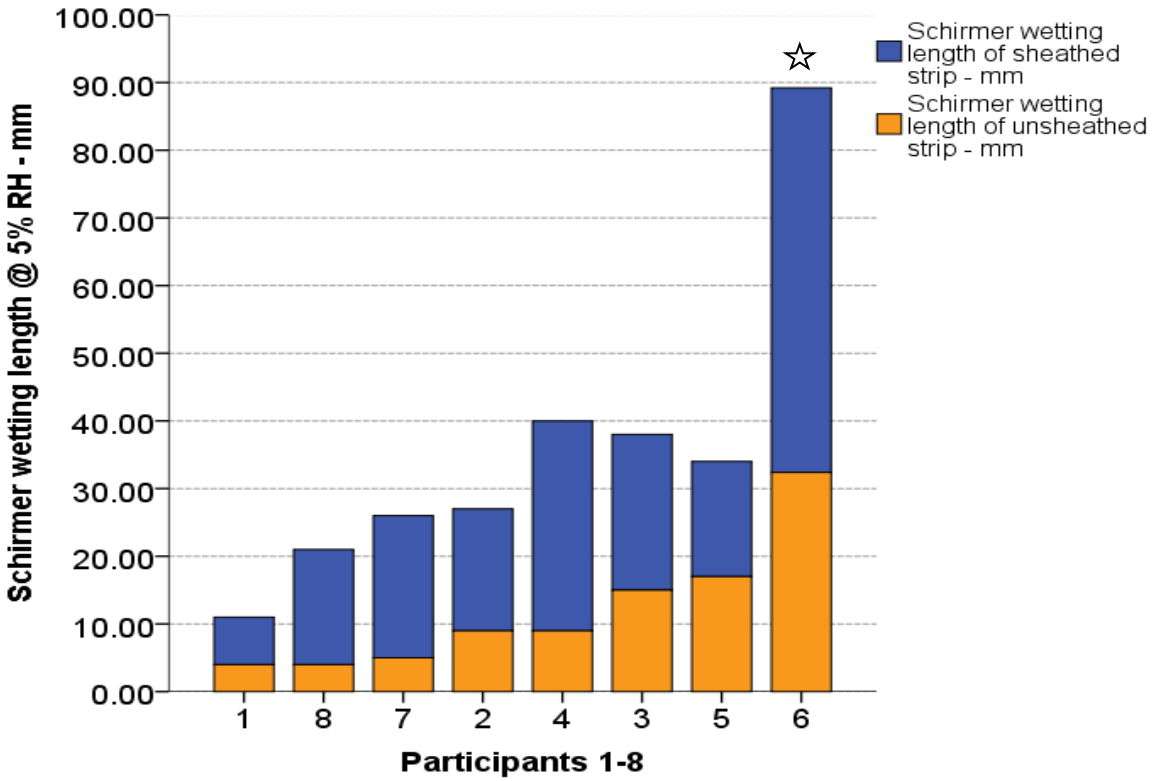


Figure 4.7 Stacked bar graph showing the Schirmer wetting length in the sheathed and unsheathed conditions of each participant at 5% RH.☆ These values represent extrapolated Schirmer value at 5 minutes and were used for samples in which the strip was completely wet before the 5 minutes.

15% Relative Humidity

The raw data of Schirmer wetting length for each participant at 15% RH can be found in Table 4.3. The mean Schirmer wetting length in the sheathed condition was 22.5 ± 13.5mm and in the unsheathed condition 12.6 ± 8.6mm (Figures 4.8 and 4.9). The mean difference in wetting length was 9.8 ± 6.7mm, representing a 44.3 % mean increase in Schirmer wetting length on the sheathed side, $t_{(7)} = 4.143$ ($p = 0.004$).

Participants (gender/age)	**1** F/20	**2** F/19	**3** F/22	**4** M/32	**5** F/21	**6** F/25	**7** M/79	**8** M/70
15% RH								
Sheathed side	R	L	R	L	R	L	L	R
Test time (seconds)	300	300	300	300	300	230	300	300
Sheathed wetting length (mm)	7	19	33	34	15	46*	11	15
Unsheathed wetting length (mm)	2	14	14	15	8	31*	9	8
Time corrected difference in wetting length (mm)	5	5	19	19	7	15*	2	7
Mean difference in wetting length ± SD (mm)	9.8 ± 6.7mm ($p = 0.004$)							
Difference in wetting length (%)	71.4	26.3	57.6	55.9	46.7	32.6	18.2	46.7
Mean difference in wetting length ± SD (%)	44.3% ± 17.8							

Table 4.3 Sheathed and unsheathed wetting length results for each participant at 15% RH.

* For statistical analysis, the extrapolated Schirmer value at 5 minutes was used for samples in which the strip was completely wet before the 5 minutes.

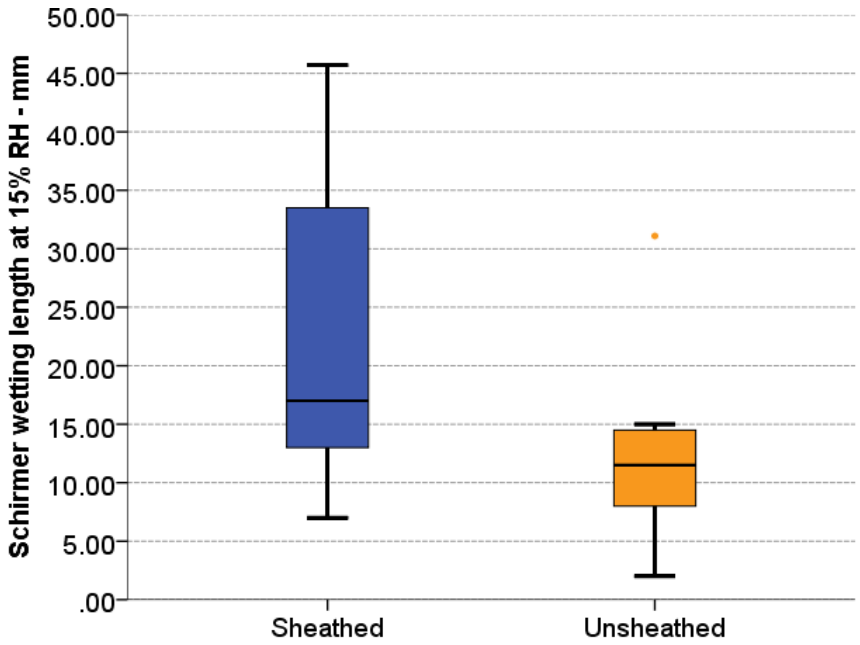


Figure 4.8 Boxplot showing the Schirmer wetting length in the sheathed and unsheathed conditions at 15% RH.

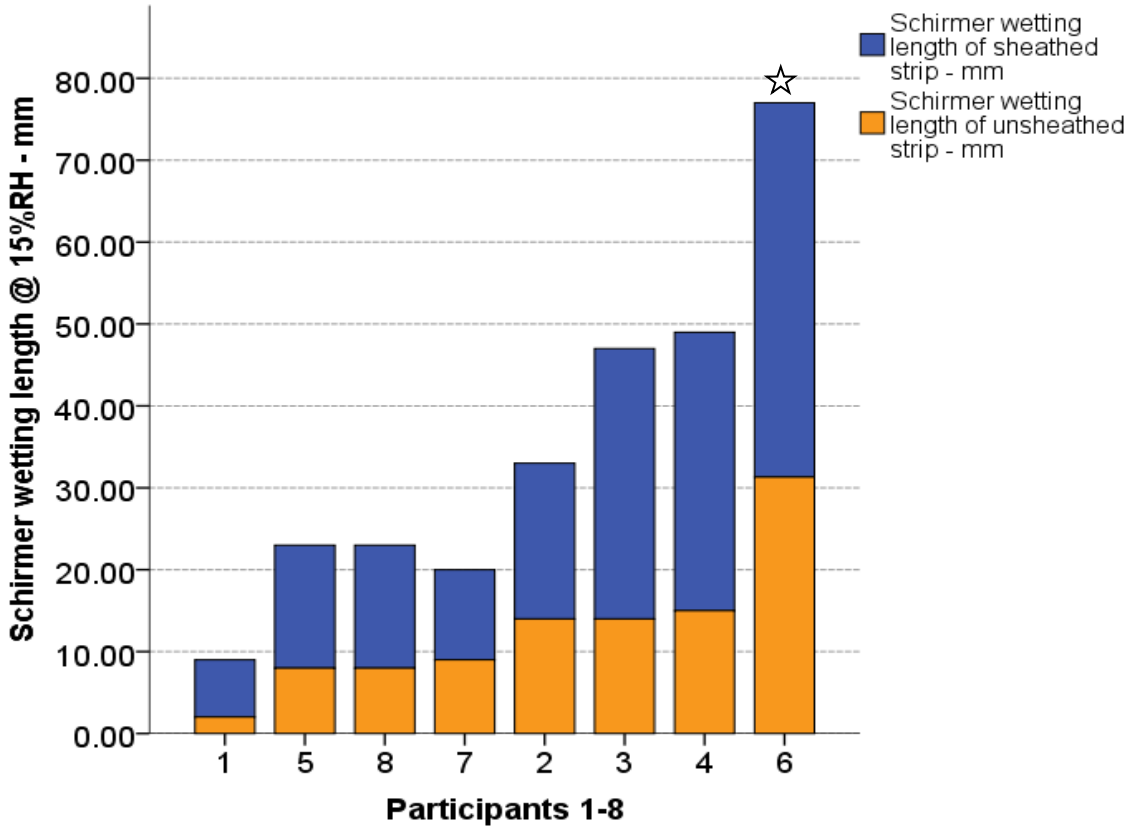


Figure 4.9 Stacked bar graph showing the Schirmer wetting length in the sheathed and unsheathed conditions of each participant at 15% RH.☆ These values represent extrapolated Schirmer value at 5 minutes and were used for samples in which the strip was completely wet before the 5 minutes.

25% Relative Humidity

The raw data of Schirmer wetting length for each participant at 25% RH can be found in Table 4.4. The mean Schirmer wetting length in the sheathed condition was 25.2 ± 11.5mm and in the unsheathed condition 15.1 ± 7.8mm (Figures 4.10 and 4.11). The mean difference in wetting length was 10.1 ± 6.0mm, representing a 39.2 % mean increase in Schirmer wetting length on the sheathed side, $t_{(7)} = 4.718$ ($p = 0.002$).

Participants (gender/age)	**1** F/20	**2** F/19	**3** F/22	**4** M/32	**5** F/21	**6** F/25	**7** M/79	**8** M/70
25% RH								
Sheathed side	L	R	L	R	R	R	L	R
Test time (seconds)	300	262	261	300	300	300	300	300
Sheathed wetting length (mm)	19	40*	40*	19	24	34	11	14
Unsheathed wetting length (mm)	8	19*	24*	10	13	28	9	9
Time corrected difference in wetting length (mm)	11	21*	16*	9	11	6	2	5
Mean difference in wetting length ± SD (mm)	10.1mm ± 6.0 ($p = 0.002$)							
Difference in wetting length (%)	57.9	52.5	40.0	47.4	45.8	17.7	18.1	35.7
Mean difference in wetting length ± SD (%)	39.2% ± 14.8							

Table 4.4 Sheathed and unsheathed wetting length results for each participant at 25% RH.

* For statistical analysis, the extrapolated Schirmer value at 5 minutes was used for samples in which the strip was completely wet before the 5 minutes.

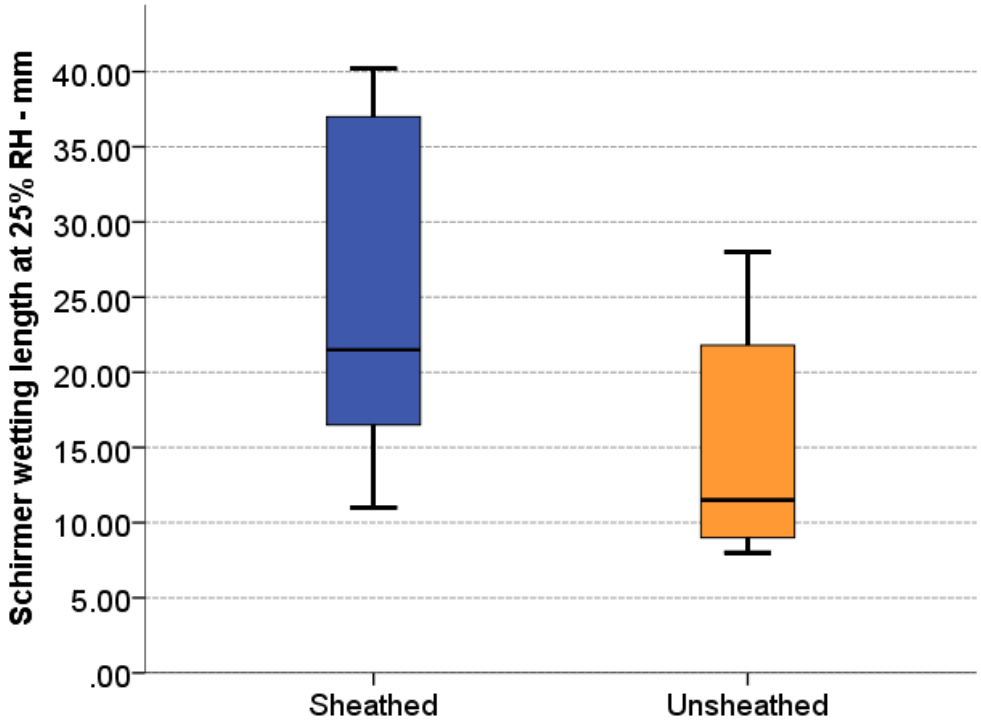


Figure 4.10 Boxplot showing the Schirmer wetting length in the sheathed and unsheathed conditions at 25% RH.

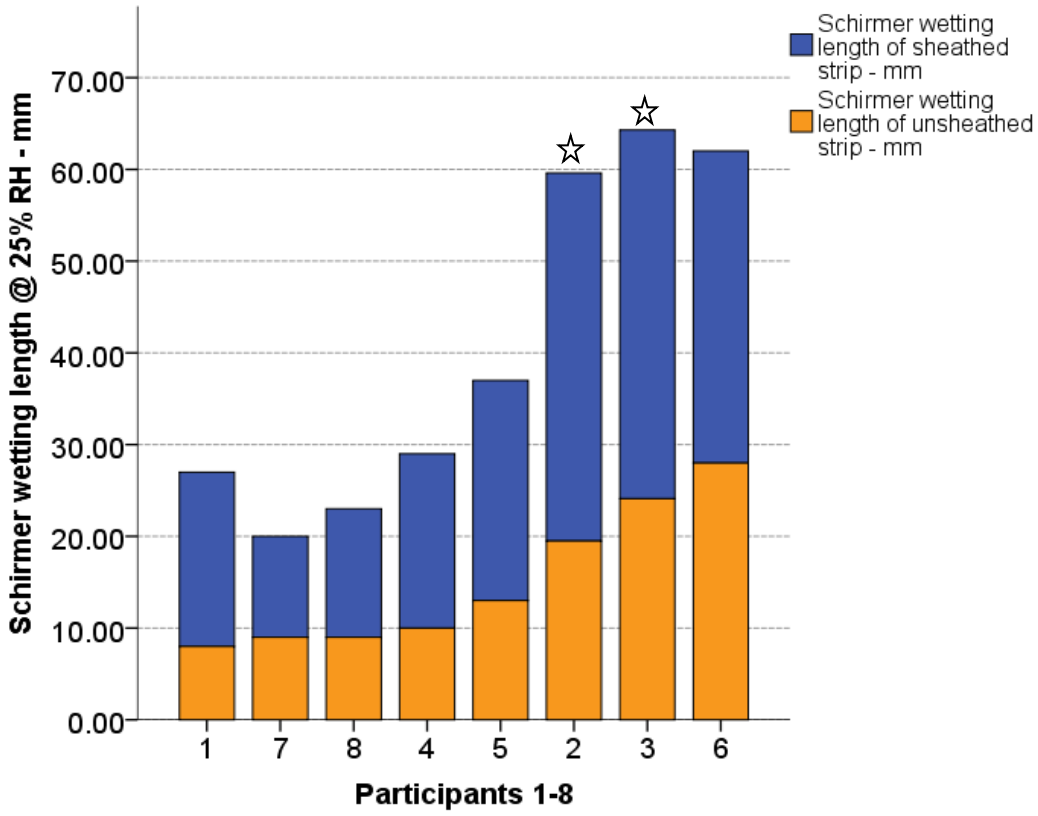


Figure 4.11 Stacked bar graph showing the Schirmer wetting length in the sheathed and unsheathed conditions of each participant at 25% RH.☆ These values represent extrapolated Schirmer value at 5 minutes and were used for samples in which the strip was completely wet before the 5 minutes.

45% Relative Humidity

The raw data of Schirmer wetting length for each participant at 45% RH can be found in Table 4.5. The mean Schirmer wetting length in the sheathed condition was 19.6 ± 6.9mm and in the unsheathed condition 12.1 ± 3.9mm (Figures 4.12 and 4.13). The mean difference in wetting length was 7.5 ± 3.8mm, representing a 36.6% mean increase in Schirmer wetting length on the sheathed side, $t_{(7)} = 5.557$ ($p = 0.001$).

Participants (gender/age)	**1** F/20	**2** F/19	**3** F/22	**4** F/32	**5** M/21	**6** F/25	**7** M/79	**8** M/70
45% RH								
Sheathed side	R	L	L	L	L	L	L	R
Test time (seconds)	300	300	300	300	300	300	300	300
Sheathed wetting length (mm)	12	10	26	17	15	25	26	26
Unsheathed wetting length (mm)	8	8	13	13	7	16	15	17
Time corrected difference in wetting length (mm)	4	2	13	4	8	9	11	9
Mean difference in wetting length ± SD (mm)	7.5 ± 3.8mm ($p = 0.001$)							
Difference in wetting length (%)	33.3	20	50	23.5	53.3	36	42.3	34.6
Mean difference in wetting length ± SD (%)	36.6 ± 11.67							

Table 4.5 Sheathed and unsheathed wetting length results for each participant at 45% RH.

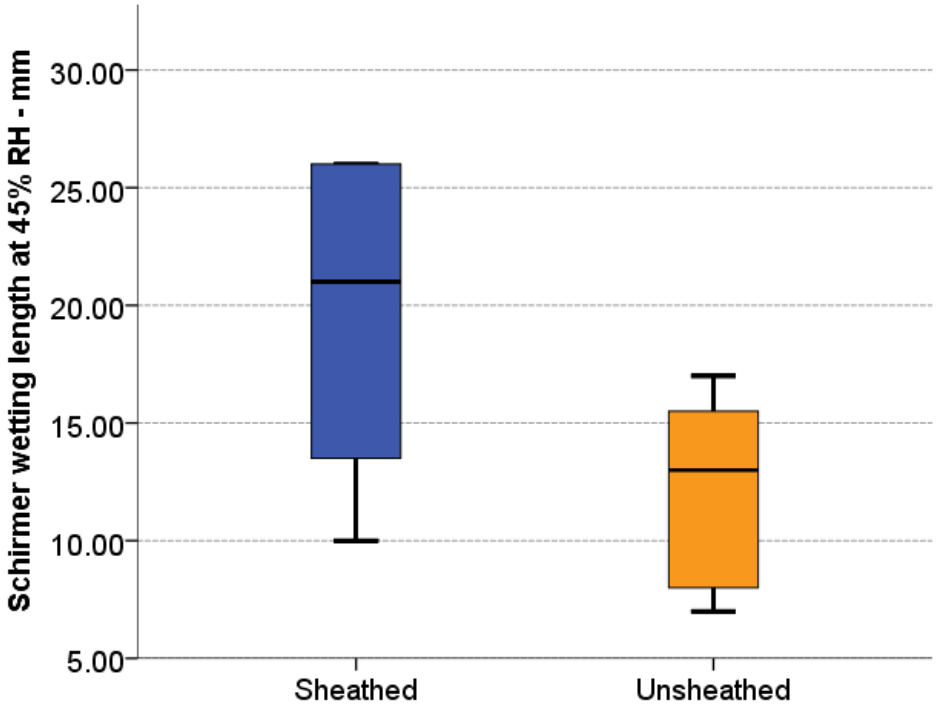


Figure 4.12 Boxplot showing the Schirmer wetting length in the sheathed and unsheathed conditions at 45% RH.

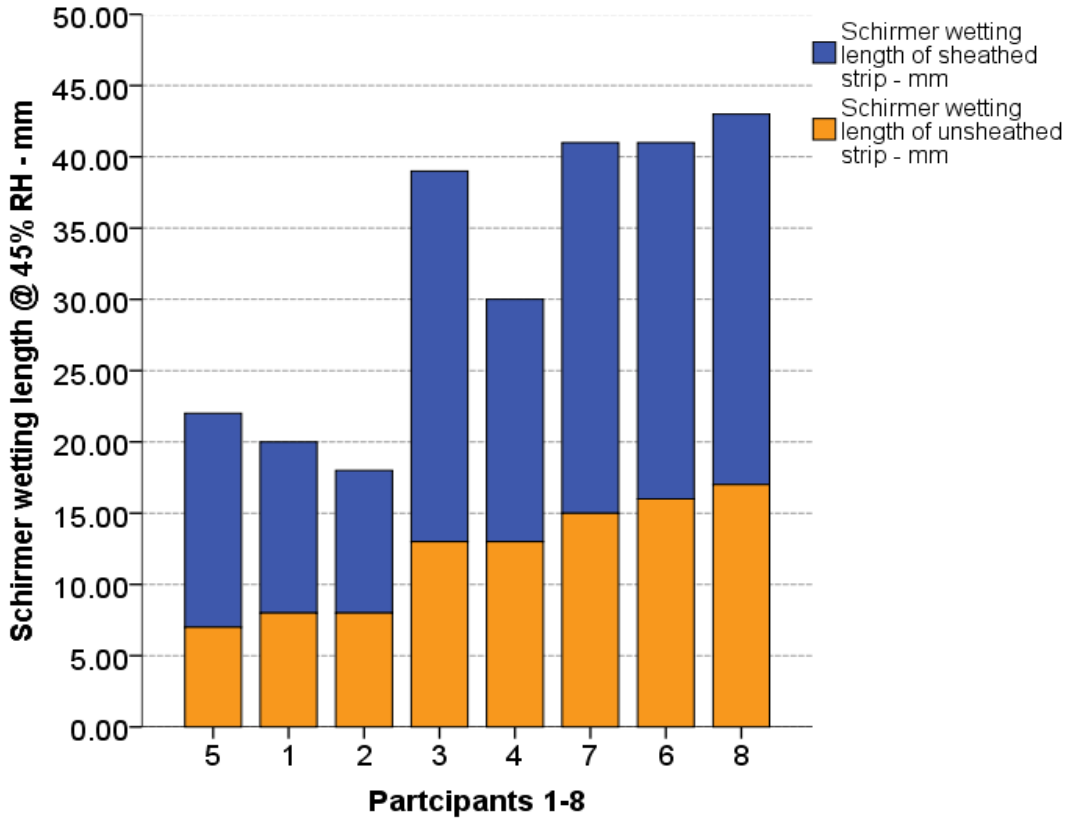


Figure 4.13 Stacked bar graph showing the Schirmer wetting length in the sheathed and unsheathed conditions of each participant at 45% RH.

Unexpectedly, no significant difference was found in the mean difference in wetting lengths between the sheathed and unsheathed conditions compared over the four different exposure levels, in other words the difference in wetting length was not demonstrated to be proportional to the level of RH, $F_{(3,21)} = 0.631$ ($p = 0.603$), although when viewed graphically a trend is discernable, indicating that the increased difference in Schirmer wetting lengths between the sheathed and unsheathed strips is found at the lower RH levels (Figure 4.14).

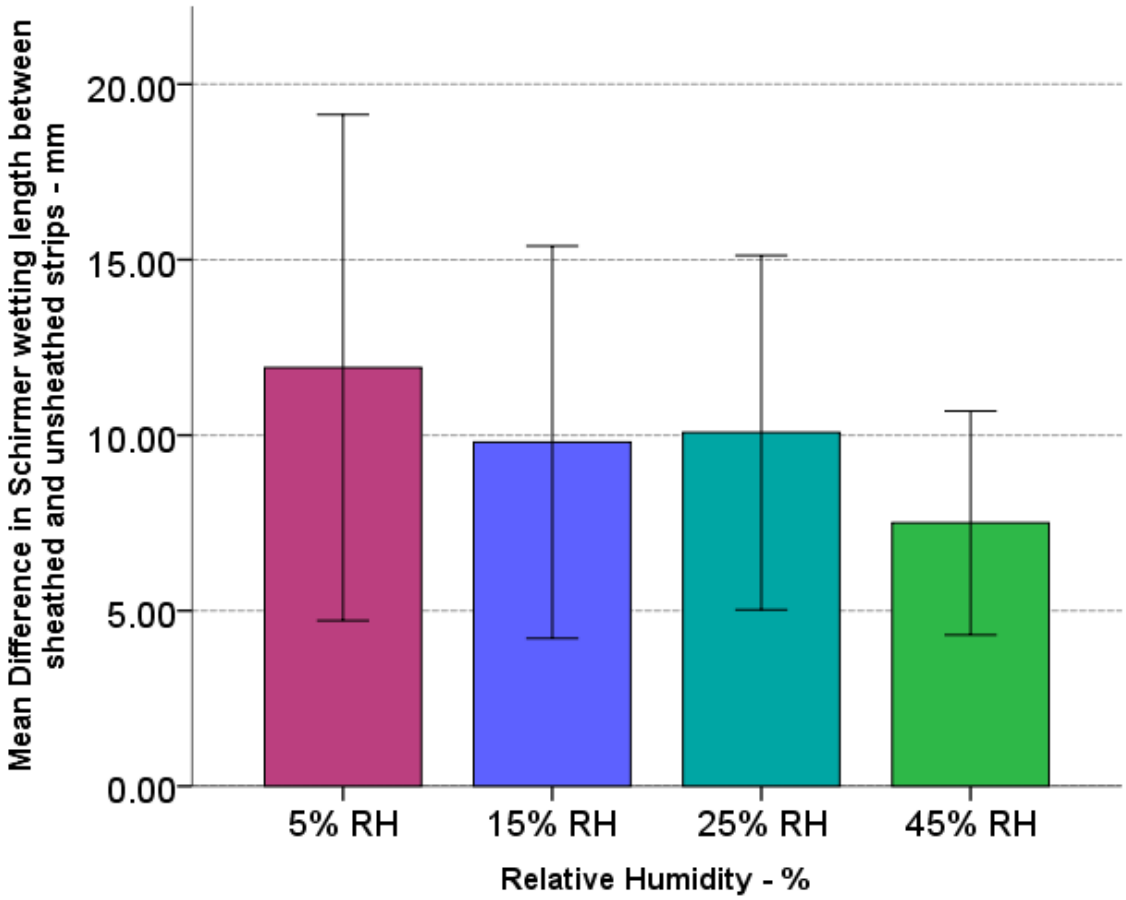


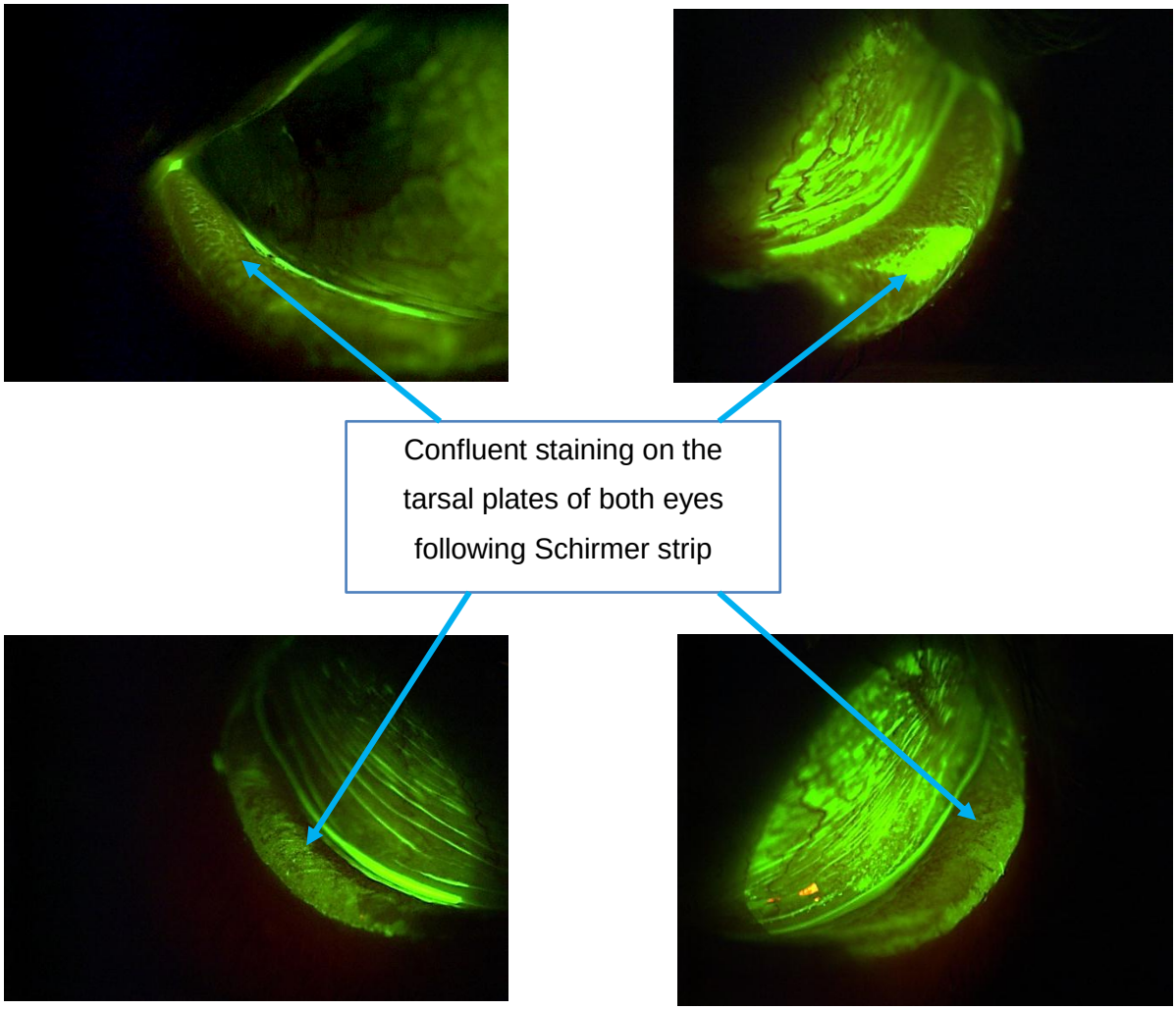
Figure 4.14 Bar graph demonstrating the mean differences in Schirmer wetting length between the sheathed and unsheathed conditions across the four different exposure levels.

4.4.1 Slit-lamp examination

Figure 4.15 shows examples of the typical appearance found in both eyes following participation in the experiment. Fluorescein examination revealed an expected confluent stain on the tarsal plates of both eyes laterally at the point of contact between the Schirmer strip and the tarsal conjunctiva. However, there was no corresponding staining on the bulbar conjunctiva. The staining did not appear to be increased in the eye that had received the sheathed Schirmer strip.

Subject 2 - RE (sheathed strip)

Subject 2 - LE (unsheathed strip)



Subject 3 – RE (unsheathed strip)

Subject 3 – LE (sheathed strip)

Figure 4.15 Examples of typical fluorescein staining of the palpebral conjuntiva following a sheathed and unsheathed Schirmer test.

4.5 DISCUSSION

The purpose of this study was to examine the influence of retarding evaporation, on the wetting length of the Schirmer strip. The wetting length was found to be greater on the sheathed side in 31/32 tests, and there was a significant difference in wetting length between the sheathed and unsheathed strips found at each level of RH tested. The study was single masked, and although each subject was masked to the location of the sheathed versus the unsheathed strip, in both sets of experiments, the investigator was fully aware of the location of the two strips.

The experiments here demonstrated here were the first to be conducted in controlled environmental conditions with a novel approach to sheathing the Schirmer strip from

evaporation. It was demonstrated that the Schirmer wetting length was greater for the sheathed Schirmer strip compared to the unsheathed at all levels of relative humidity. At 5% RH the mean difference in wetting length was 11.9 ± 8.6mm (p = 0.006); at 15% RH the mean difference in wetting length was 9.8 ± 6.7mm (p = 0.004); at 25% RH the mean difference in wetting length was 10.1 ± 6.0mm (p = 0.002) and at 45% RH the mean difference in wetting length was 7.5 ± 3.8mm (p = 0.001). These results are presented graphically in Figure 4.14. In interpreting these results, an assumption is made that the Schirmer Ia (without anaesthesia) secretory response, in the unclad state, is similar between the two eyes (Kallarackal et al., 2002). Any further studies could be adjusted using a crossover design to account for a variable flow rate between the two eyes.

Earlier studies by Holly et al., (Holly, Lamberts and Esquivel, 1982; Beebe, Esquivel and Holly, 1988; Holly, 1994) had indicated a relationship between water loss from the strip and wetting length, such that, the greater the evaporative loss, the greater the decrease in wetting length. A similar observation was made, more recently, by Buckmaster and Pearce (Buckmaster and Pearce, 2016). In this study an *in vitro* experiment was set up whereby the first 5mm of an unsheathed Schirmer strip was immersed in an unlimited water supply; the benefit of this approach was that it allowed pure observation of the effects of RH on Schirmer wetting since the influence of reflex tearing and biological variation was removed. Measurements of wetting length after 5 minutes at RHs of 5%, 20%, 35%, 50%, 65%, 80% and 95% and a temperature of 21°C were taken, using six Schirmer strips per RH level. Results showed a significant decrease in wetting length as the RH reduced, ranging from 27mm at 5% to 39mm at 95%. In a subsequent experiment the Schirmer strips were modified to prevent evaporation from the strip using a “plastic film” that started at the 10mm notch and the experiments then repeated as above. It was reported that in the sheathed condition the wetting length did not change at any of the RH levels. Lastly an *in vivo* study was also conducted. Ten normal subjects received both bilateral unsheathed and sheathed Schirmer tests at RHs of 20%, 50% and 80%. There was a significant reduction in wetting length in the unsheathed Schirmer strip demonstrated between the 20% and 80% RH conditions, and a much lower variation in wetting lengths when the Schirmer strips were sheathed. The overall conclusion was that in lower levels of Schirmer wetting lengths, such as those expected in DED, the effect of evaporation was negligible. However, in those patients with borderline wetting lengths ~10mm, there was the potential that they could be falsely diagnosed with DED, since these measures are often conducted in conditions of low humidity. A recent poster presented at ARVO re-asserted the usefulness of the Schirmer Tear Test (STT), stating that with some adaptation to the methodology, and by enclosing the Schirmer strip in a water-proof sheath (transparent tape), it could provide a reliable, quantitative measure of tear production and aid in the differential diagnosis of ADDE and EDE (Radke et al., 2017). Results from this study indicated that, providing an initial phase of non-linear

wetting, due to the uptake of the tears present on the lid margin, was discounted, the following period of wetting that occurred at a much slower, linear rate, would allow the tear production rate to be calculated. However, no comparison was made between a sheathed and unsheathed Schirmer wetting length condition. The study endorsed the importance of sheathing the strip to obtain a more valid statement for the basal (anaesthetic) tear production and to obtain the best clinical results by reducing evaporation from the strip. A similar conclusion had been drawn in the earlier work of Holly et al., (Holly, Lamberts and Esquivel, 1982).

The effect of evaporation on the Schirmer wetting length described here and in the studies of Buckmaster and Pearce (Buckmaster and Pearce, 2016), and Radke et al., (Radke et al., 2017) was probably responsible for a paradoxical finding in a recent study, by Abusharha and Pearce (Abusharha and Pearce 2013). It was found that exposure of normal subjects to prolonged desiccating stress, at a RH of 5%, caused, as expected, a marked increase in tear evaporation compared to that measured at 40% RH, together with symptoms of increased discomfort. Increased discomfort could be explained by surface cooling and a rise in tOsm, due to the increased evaporation. However, a normally operating LFU in these subjects would be expected to offset these effects of increased evaporation by reflexly stimulating a rise in lacrimal secretion (Stern et al., 2004). This would account for the finding that although the average tOsm was higher at 5% RH, it was not significantly different from that at 40% RH. Surprisingly however, the Schirmer test value, used to measure the reflex tear secretory response at 5% RH at the end of the study, was *reduced* rather than *increased*, which conflicted with the prediction of a reflex increase in lacrimal secretion. This result was most likely due to an artefact of measurement, caused by excessive evaporation from the Schirmer strip exposed at 5% RH. It may be concluded that in those experiments, desiccating stress did indeed result in homeostasis of tOsm, due to the stimulation of lacrimal secretion, but that the lacrimal secretory response was masked by an artefact in the Schirmer test measurement. In other words tOsm would have risen, were it not for a secretory response of the lacrimal gland. As mentioned in the introductory chapter this evaporative artefact could also be responsible for similar, anomalous tear flow and tOsm responses to low RH reported by Teson et al., (2013) and López-Miguel et al., (2014) in subjects exposed to 5% RH for a period of two hours (Teson et al., 2013; López-Miguel et al., 2014).

An important issue of interpretation needs to be dealt with here. In the above studies and in the work reported in this chapter, the Schirmer response, say at 5% RH, is spoken of as if it were reporting the lacrimal response to the exposed relative humidity, perhaps having reached a new steady state at the time of measurement. Actually, that value can only be captured by a minimally-invasive technique such as fluorophotometry, or indirectly by

inferring changes in tear volume from a measurement of tear meniscus volume. It is not certain that the reflex Schirmer test (Ia) referred to here will tap into and reflect this effect. Therefore, in the study reported here, the Schirmer test is being used as a test-bed to explore the influence of sheathing on the recorded response, rather than to make a statement about the lacrimal response to different RH levels. In the current study, although there was a visual trend towards an increased difference in wetting lengths with decreasing RH, the relationship was not significant (p = 0.603). Several possible reasons for this may be considered, including the small numbers of subjects in the study and the impact of repeated Schirmer tests on individual responses in one session, which was not predictable in terms of the effect of one Schirmer test on a subsequent one. Unrecognised variations in permeability of the sheathing may have occurred and local differences in air currents and other environmental factors may have operated in the neighbourhood of the Schirmer strips and been responsible for the unexpected findings. There are other factors that need to be taken into account in this study such as the fact that the sheathing of the Schirmer strip added extra weight resulting in the strip sitting closer to the cheek when compared to the unsheathed strip (Figure 4.16). This could influence the final wetting result since gravity may have added to the speed of wetting. However, in a previous experiment by Holly et al., (1982) the difference in wetting length contributed by gravity was found to be “barely detectable” (Holly, Lamberts and Esquivel, 1982). In a recent modelling paper by Telles et al., (2017), gravity is stated to increase wetting progression by approximately 10%, but only after the wetting front reaches the point at which the strip is bent for insertion into the eye, being negligible due to imbibition forces, before this point. Eliminating the consideration of gravity from mathematical calculations still resulted in a tear production rate of 1.19 µL/min, a value still in line with previous estimates (Mishima et al., 1996: Cerretani and Radke, 2014).

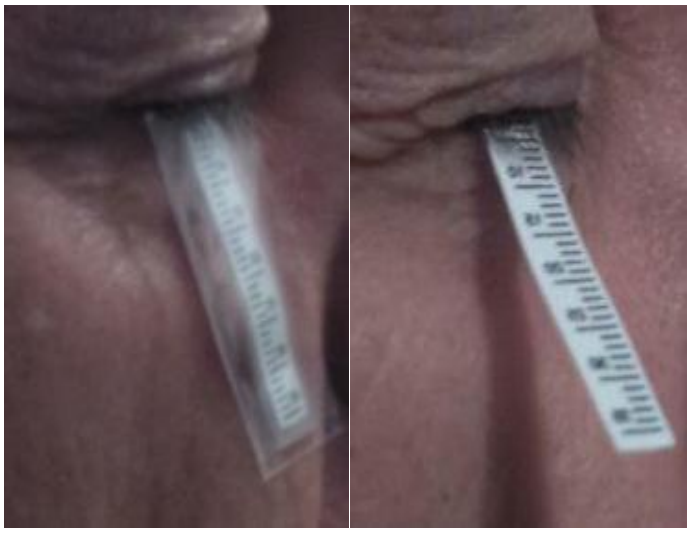


Figure 4.16 Position of a sheathed and unsheathed Schirmer strip once in situ.

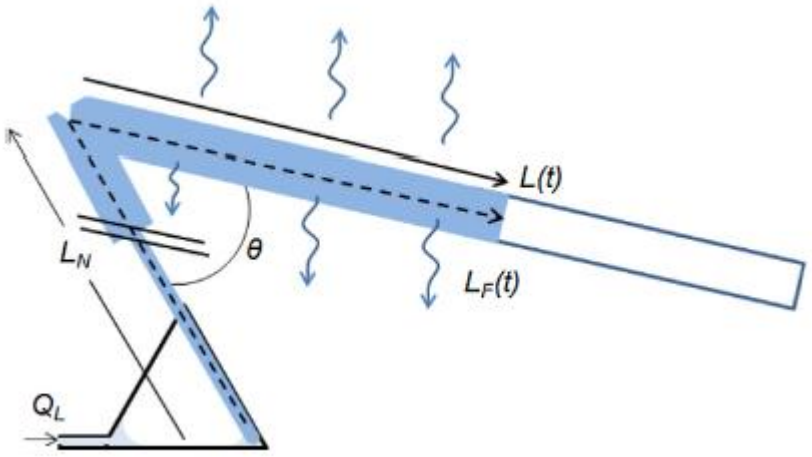


Figure 4.17 Schematic of a Schirmer strip inserted into the tear lake shown in cross section as an equilateral triangle. Curved lines with arrows indicate tear evaporation. From Telles et al. (2017). *Colloids Surf Physicochem Eng Aspects* **521**:61-68.

It is also possible that evaporation from the Schirmer strip may have taken place to a greater extent on the superior surface. The inferior surface may have been protected from evaporation by a possible pocket of increased humidity forming between the paper and the cheek. Modelling has demonstrated that evaporation can occur from the top and bottom of the Schirmer strip (Figure 4.17) once wetting emerges from the inferior fornix, slowing the progression of the wetted front (Telles et al., 2017). The sheath created for this study was assumed to be completely waterproof; however this was not tested and any deviation from this state could have contributed to an increased evaporation from the Schirmer strip.

Airflow, temperature and relative humidity in clinic conditions can vary in different locations and environments and air-conditioning could play a negative role, influenced, for instance by subject placement within a room. A wider variation in ambient conditions could exist on a global basis where, in the absence of air-conditioning, subjects could be exposed to seasonal variations in ambient conditions (Teson et al., 2013; López-Miguel et al., 2014). With these ambient conditions in a clinical setting varying from day-to-day, the effect of the phenomenon first described by Holly et al., (1984) on the results of the Schirmer test could dramatically influence the diagnosis of DED or be misleading for the development of treatment plans (Holly, Laukaitis and Esquivel, 1984). Standardising the different RH conditions in this study was made possible by the use of the CEC and was the first study to employ this equipment in this context.

To conclude, the current study (along with the work of Buckmaster and Pearce, 2016; Radke et al., 2017) supports the original work by the Holly group (Holly, Lamberts and Esquivel, 1982; Beebe, Esquivel and Holly, 1988; Holly, 1994), and suggests that in all

situations, particularly any study using the Schirmer test to examine the dynamic response of the LFU to changes in the physical environment, the strips should be sheathed, in order to demonstrate the true reflex secretory response to desiccating conditions and to free the test from a dependence on ambient conditions that can vary seasonally, geographically and locally from clinic to clinic.

Chapter 5:

ESTIMATING BASAL TEAR OSMOLARITY IN NORMAL AND DRY EYE SUBJECTS

These experiments were designed to ascertain whether it is possible to drive down tear osmolarity (tOsm) to a basal level by suppressing tear evaporation, with the prediction that such a value should lie close to that of plasma osmolality (pOsm). In this study, both normal and dry eye disease (DED) subjects were assessed, using two different methods to suppress tear evaporation.

5.1 INTRODUCTION

Tear osmolarity (tOsm) measured from the tear meniscus is composed mainly of lacrimal fluid with lesser contributions from the conjunctiva and cornea, the degree of which is currently unknown. This tear sample, routinely used as a diagnostic tool for DED, is assumed to be higher than that of the secreted tears as a result of evaporation from the ocular surface induced by exposure to ambient environments (Mishima et al., 1966; Mishima, 1965; Mishima and Maurice, 1961a; b).

A fluctuation in tOsm levels has been shown to occur throughout the day. Niimi et al., (2013) measured tOsm levels over an eight hour period in 38 normal subjects; Table 5.1 shows the recorded levels of tOsm measured up to 8 hours following a minimum period of six hours sleep (Niimi et al., 2013).

Time	Tear Osmolarity (mOsm/L)
20 minutes	287 ± 10
40 minutes	292 ± 16
1 hour	293 ± 12
2 hours	292 ± 10
4 hours	289 ± 10
8 hours	286 ± 10

Table 5.1 Mean tOsm over an 8 hour period. From Niimi et al. (2013). *Cornea* **32**(10): 1305-1310.

Variation in tOsm demonstrated over the course of the day is reported to be more pronounced as the severity of the DED condition increases. Sullivan et al., (2012) monitored DED subjects over a period of three months and showed tOsm varied by 5.9 mOsm/L ± 3.1% in mild DED (n=16) and by 10.0 mOsm/L ± 6.9% in severe DED (n=36) (Sullivan et al., 2012).

Ambient conditions are responsible for evaporation, including personal behaviour e.g. VDU use, and can account for the variation of tOsm over the course of the day, and measurement of tOsm in uncontrolled conditions of humidity, temperature and airflow invites variation of tOsm and this effect is amplified in DED (Sullivan, Pepose and Foulks, 2015). This effect is intensified when the eyes are exposed to desiccating stress, such as low humidity, high temperature and high airflow (Gonzalez-Garcia et al., 2007; Madden, Tomlinson and Simmons, 2013; Teson et al., 2013; Peng et al., 2014; López-Miguel et al., 2014).

As noted in a few papers, tOsm has been shown to decrease following a period of eye closure, as in sleep, attributed to the suppression of evaporation. Niimi et al., (2013) found tears to be significantly hypo-osmotic after a period of sleep: 264 ± 14 mOsms/L, compared with the pre-sleep value of 297 ± 15 mOsms/L (Niimi et al., 2013). Terry and Hill (1978) reported the average daytime tear osmolarity in 6 young adults to be 310 ± 5.7 mOsm/Kg (range 299-323 mOsm/Kg) which compared to a tOsm of 285 ± 2.4 mOsm/Kg (range 282-288 mOsm/Kg), measured immediately after a 6-8 hour period of sleep (Terry and Hill, 1978). In the latter study the closed eye tOsm values, in given individuals, also showed less variability.

The term basal tears was created for this study and refers to the mixture of lacrimal, conjunctival and cornea fluid predicted to exist with continued secretion, mixing and drainage of tears; and following a period of evaporative suppression wherein a state of equilibration occurs with the interstitial fluid across the ocular surface epithelia, and tOsm falls to levels close to that of the plasma.

In a study by Fortes et al., (2011) a positive relationship between whole body hydration an tOsm was reported, measured as pOsm and tOsm, in a group of young adults during a period of imposed systemic dehydration and during restoration to euhydration (Fortes et al., 2011). Using a tOsm reference value of 310 mOsm/L this estimated a sensitivity of 80% and a specificity of 92% using this approach for the detection of sub-optimal hydration (Fortes et al., 2011).

A problem arises in adopting the novel proposal of Fortes et al., (2011) to detect sub-optimal body hydration in the elderly by means of measuring tOsm in open-eye conditions (Fortes et al., 2011). Since the prevalence of body dehydration rises with age (Xiao, Barber and Campbell, 2004), as does the prevalence of dry eye (Schein et al., 1997), and hence that of tear hyperosmolarity due to local causes. There is an increased likelihood of misdiagnosis of body dehydration with increasing age.

A proposed adaptation to circumvent this difficulty is that of evaporative suppression achieved by either a period of eye closure (as in sleep), or by exposure of the open eyes

to an environment of high humidity (as with humidity goggles). In these conditions it is anticipated that tOsm would be driven down to a basal level that would be individual to a given person and be subject to less variation than open-eye tOsm and represent a level of tOsm close to pOsm.

5.2 HYPOTHESIS

It was hypothesised that in the absence of tear evaporation and with continued tear secretion, mixing and drainage, the osmolarity of the tears, equilibrating with the interstitial fluid across the ocular surface epithelia, would fall to a basal level close to that of the plasma.

It was predicted that this value, termed here the basal tear osmolarity (BTO), would show less variation than the tOsm measured in open eye conditions and would be individual to the subject and independent of the tOsm starting point. Thus the presence of tear hyperosmolarity of *any* degree would not influence the final BTO value. If these conditions prevailed, then it was expected that the BTO would be a better surrogate for pOsm than tOsm measured in open eye conditions and could serve as a useful diagnostic index of water-loss dehydration in the elderly. Additionally, in DED, the differential between the BTO and the level of tear hyperosmolarity in that patient should provide a more accurate guide to DED severity.

5.3 MATERIALS AND METHODS

5.3.1 Subject enrolment

Eight subjects with normal eyes were recruited, 4 male and 4 female, aged 30.88 years ± 10.49 (mean ± SD) from students and staff at Anglia Ruskin University. Participants were individuals with a normal ocular surface by history and examination, according to defined criteria (see Chapter 2 for screening inclusion/exclusion criteria).

Eight subjects with DED were recruited, 1 male and 7 female (4 diagnosed with Sjögren Syndrome), aged 55.75 years ± 17.05 (mean ± SD) from staff at Anglia Ruskin University and from the Cambridgeshire British Sjögrens Syndrome Association (BSSA) group. DED participants were individuals who fulfilled the internationally accepted definition of Dry Eye Disease (Lemp et al., DEWS I 2007) by history and by ocular surface examination (see Chapter 2 for screening inclusion/exclusion criteria). Table 5.2 displays the recruitment credentials of the normal and DED subjects.

Parameter Mean ± SD (min-max)	Subjects		
	Normal (n=8)	Sjögren syndrome DED (n=4)	Non Sjögren syndrome DED (n=4)
Gender	4 Male : 4 Female	4 Female	1 Male : 3 Female
Age (years)	30.88 ± 10.49 (20-53)	66.75 ± 7.93 (55-72)	48.25 ± 18.73 (24-67)
tOsm (mOsm/L)	293.8 ± 6.34 (285-301)	314.3 ± 11.21 (308-331)	313 ± 5.42 (309-321)
Corneal staining	0.13 ± 0.35 (0-1)	3.5 ± 2.38 (2-7)	2.0 ± 0.0 (2-2)
Schirmer wetting (cm)	20.3 ± 2.71 (17-24)	5.3 ± 2.5 (2-8)	6.0 ± 1.83 (4-8)
OSDI	5.08 ± 6.07 (0-15)	64.9 ± 25.49 (43.75-95)	41.9 ± 22.42 (27.08-75)
TBUT (seconds)	11.35 ± 0.94 (10.24-12.83)	2.39 ± 0.67 (1.44-3.01)	4.99 ± 1.15 (3.57-6.34)
Meibography grading	0 ± 0.0 (0-0)	1 ± 0.0 (1-1)	0.5 ± 0.58 (0-1)

Table 5.2 Profiles of the DED and normal subjects at the screening visit

All subjects were instructed not to use ocular cosmetics on the day of assessment, and if contact lens wearers, not to use their lenses for at least 8 hours before the procedure; they were also asked not to use artificial tears for at least 4 hours prior to data collection.

5.3.2 Equipment

A controlled environment chamber (CEC) was employed for the two visits made by the subjects on separate days. In this study experiments were conducted in two different controlled, environmental conditions: i. 'Standard room' conditions and, ii. 'Evaporative Suppression' conditions. Tear osmolarity was also routinely measured in all subjects prior to entry into the CEC, in uncontrolled environmental conditions, in the clinic area immediately outside the chamber. These tOsm measurements are referred to as taken in 'clinic conditions' (Table 5.3). Actual room temperature and RH% were recorded at the time of these measurements.

	Temperature	Relative Humidity	Airflow
'Standard room' conditions	23°C	45%	0.08m/s
'Evaporative Suppression' conditions	23°C	70%	0.08 m/s
'Clinic conditions'	Uncontrolled environment: temperature and relative humidity recorded at each visit		

Table 5.3 Defined environmental conditions used in the current study.

After the subject and examiner had entered the CEC, a period of 10 minutes was allowed to pass to allow for equilibration. A slit-lamp examination was carried out following each visit to check for adverse events. The TearLab® machine was modified for this

experiment (see Chapter 2) so that low osmolarity values (below 270 mOsm/L) which would ordinarily trigger a “Below Range” error message could be read.

5.3.3 Protocol

The eye closure visit lasted approximately 90 minutes. Once seated in the CEC (set to standard room conditions) the subject was provided with a set of headphones playing classical music and when comfortable was asked to close the eyes for a period of 45 minutes, during which time they were encouraged to keep the eyes moving behind closed eyelids to facilitate tear mixing. After 45 minutes the subject was asked to open the eyes and tOsm was measured, first in the right then in the left eye, immediately on eye opening. The subject was then instructed to keep both eyes open (exposed to standard room conditions), blinking spontaneously for a further 45 minutes, over which time tOsm was measured in each eye, first on the right and then the left, at 15 minute intervals.

The exposure to 70% RH visit occurred on a subsequent day and lasted approximately 45 minutes. After entering the CEC (set to high humidity conditions) the subject was seated and instructed to keep the eyes open, blinking spontaneously for 45 minutes over which time tOsm was measured in each eye, first on the right and then the left, at 15 minute intervals, as in the first session. See Figure 5.1 for a timeline of the experiments and Appendix IX for a sample record card.

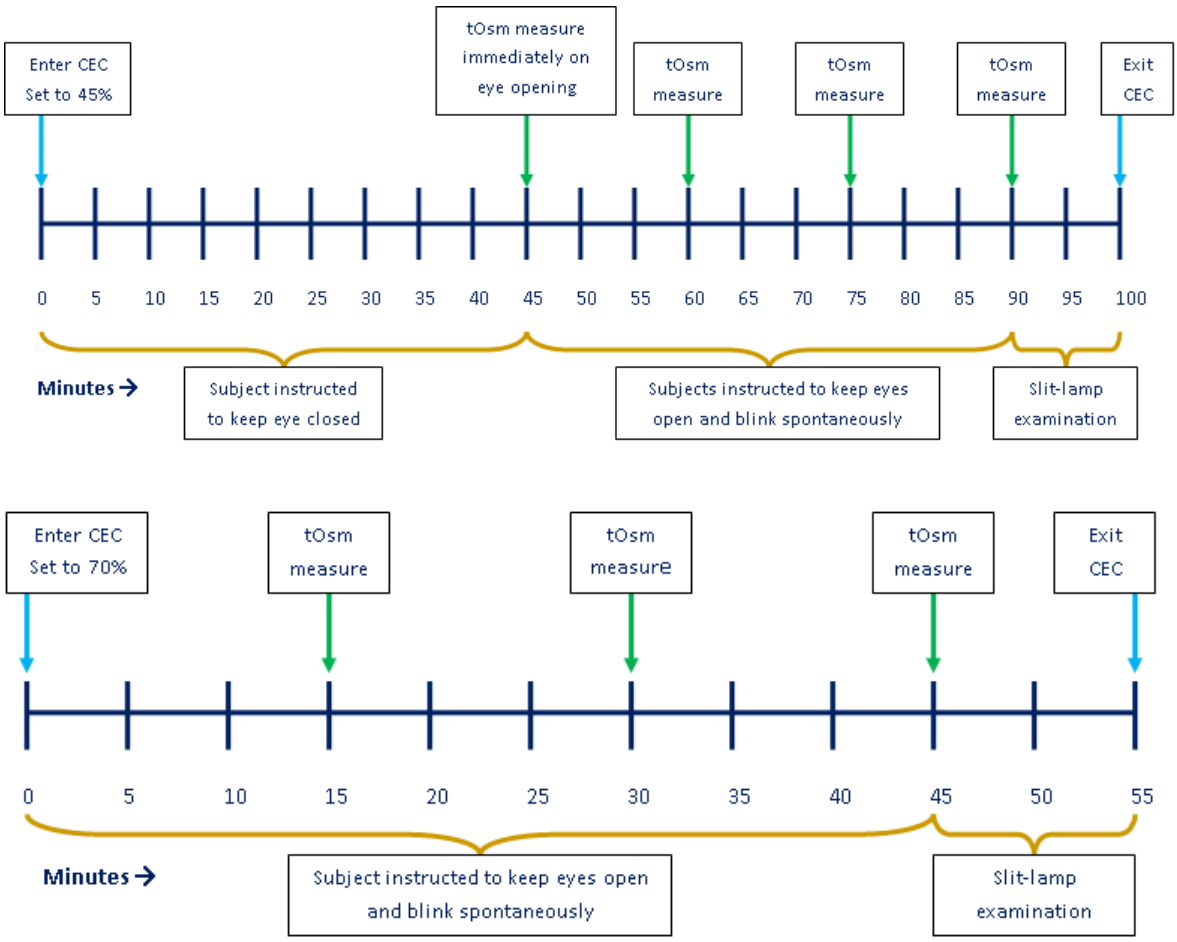


Figure 5.1 Timeline of eye closure and exposure to 70% RH visits.

5.3.4 Statistical analysis

The change in mean tOsm measured in clinic conditions from that measured after eye closure or at the end of 45 minutes exposure to 70% RH, was analysed independently for right (RE) and left eye (LE) and, additionally, averaged between the two eyes (BE) (as in the study of Holland et al., 2017).

The effect of eye closure

In the normal group (RE and LE individually), differences in tOsm were compared using a one-way repeated measure ANOVA.

In the DED group, differences in tOsm were compared using a one-way repeated measure ANOVA for the RE and a non-parametric Friedman test for the LE (with post hoc testing due to 3 outliers).

When the average tOsm values for both eyes (BE) were used, the changes in tOsm in the normal and DED groups were compared using a one-way repeated measure ANOVA in both instances.

The effect of 70% relative humidity

In the normal group (RE and LE individually), differences in tOsm were compared using a one-way repeated measure ANOVA. In the DED group (RE and LE individually) differences in tOsm were compared using a non-parametric Friedman test (with post hoc testing as 1 outlier in the RE and 2 outliers in the LE). When the tOsm values for BE were averaged, the changes in the normal group were compared using a one-way repeated measure ANOVA and with a non-parametric Friedman test in the DED group (with post hoc testing as 2 outliers).

Differences in the effects of eye closure and 70% relative humidity

Differences in tOsm measured between the two conditions of evaporative suppression in the normal group (RE and LE individually and BE averaged) (45 minutes of eye closure and 45 minutes of exposure to 70% RH) were compared using a paired t-test and in the DED group RE individually and BE averaged using the non-parametric Sign test (as 2 outliers and data not symmetrically distributed) and LE individually using a non-parametric Wilcoxon signed-rank test (as 1 outlier).

Inter-eye differences in tOsm for the normal and DED groups in uncontrolled environment conditions and after evaporative suppression (eye closure and after 45 minutes in 70% RH) were compared using a paired t-test.

A Shapiro-Wilk test was used to evaluate normality of distribution. Differences were considered statistically significant for *P* values less than 0.05. Data is presented as mean ± standard deviation. Analyses were performed using SPSS 20.

5.4 RESULTS

Details of the clinic conditions for all 32 experiments (16 experiments for the normal group and 16 experiments for the DED group) are presented in Table 5.4. The temperature fluctuated between 16°C and 27°C, and the RH between 20% and 69% during tOsm measurements prior to CEC entry in clinic conditions, for the normal and DED groups. These measures were recorded to show the variation encountered in the uncontrolled clinic environment on a day to day basis, compared to those created in the controlled environment of the CEC, or during eye closure.

Experiment Number	Temperature (°C)		Relative Humidity (%)	
	Normal	**DED**	**Normal**	**DED**
1	24	21	22	21
2	23	23	20	24
3	21	23	21	39
4	23	26	30	27
5	24	23	28	23
6	23.3	27	62	48
7	22	22.4	41	62
8	22.3	22.7	63	41
9	16	21	37	63
10	18	23	29	55
11	22	23	31	63
12	24	24	33	65
13	25	24	28	69
14	22.7	26	55	56
15	22	21.9	63	67
16	22	22.9	65	57
Range	**9.0**	**6.0**	**45.0**	**49.0**
Min/Max	**16°C / 25°C**	**21°C / 27°C**	**20% / 65%**	**20% / 69%**

Table 5.4 Temperature and RH levels recorded in the uncontrolled, clinic conditions at the time of tOsm sampling in each subject.

The tOsm values for the normal and DED subjects in the two different environments of evaporative suppression are presented in Tables 5.5-5.8.

Subject	tOsm clinic conditions (mOsm/L)			tOsm on eye opening (mOsm/L)			tOsm 15 minutes (mOsm/L)			tOsm 30 minutes (mOsm/L)			tOsm 45 minutes (mOsm/L)		
	RE	LE	BE	RE	LE	BE	RE	LE	BE	RE	LE	BE	RE	LE	BE
1	288	293	290.5	287	285	286	289	300	294.5	300	294	297	296	297	296.5
2	290	289	289.5	290	291	290.5	300	290	295	309	288	298.5	289	289	289
3	289	293	291	283	280	281.5	292	292	292	293	289	291	292	295	293.5
4	295	298	296.5	283	286	284.5	303	295	299	296	309	302.5	303	301	303.5
5	293	282	287.5	290	282	291	297	296	296.5	305	309	307	296	*	*
6	297	299	298	286	277	281.5	297	287	292	291	299	295	292	292	292
7	292	294	293	285	288	286.5	287	291	289	289	285	287	288	282	285
8	299	297	298	286	288	287	286	295	295.5	295	299	297	301	293	297
Mean ± SD (range)	**292.9 ± 2.91 (11)**	**293.1 ± 5.54 (17)**	**293.0 ± 4.05 (10.5)**	**286.3 ± 2.71 (7)**	**285.9 ± 5.16 (15)**	**286.5 ± 3.57 (9.5)**	**295.1 ± 5.44 (16)**	**293.3 ± 4.06 (13)**	**294.2 ± 3.10 (10)**	**297.3 ± 6.94 (20)**	**296.5 ± 9.20 (24)**	**296.9 ± 6.24 (20)**	**294.6 ± 4.0 (15)**	**293.5 ± 6.39 (22)**	**294.1 ± 5.60 (18.5)**

* Represents a discarded reading due to difficulty in sampling

Table 5.5 Tear osmolarity values for **Normal** subjects following 45 minutes of eye closure and 45 minutes exposure to 45% RH

Subject	tOsm clinic conditions (mOsm/L)			tOsm 15 minutes (mOsm/L)			tOsm 30 minutes (mOsm/L)			tOsm 45 minutes (mOsm/L)		
	RE	LE	BE	RE	LE	BE	RE	LE	BE	RE	LE	BE
1	309	307	308	301	299	300	299	297	298	299	296	297.5
2	304	302	303	299	293	296	297	290	293.5	295	292	293.5
3	291	287	289	298	291	294.5	297	291	294	298	300	299
4	294	286	295	299	294	296.5	296	296	296	296	298	297
5	301	304	302.5	303	300	301.5	299	293	296	298	296	297
6	308	311	309.5	301	296	298.5	303	296	299.5	302	296	299
7	283	296	289.5	286	292	289	285	293	289	285	293	289
8	300	299	299.5	292	290	291	290	285	287.5	289	286	287.5
Mean ± SD (range)	**298.8 ± 8.91 (26)**	**300.3 ± 7.48 (24)**	**299.5 ± 7.78 (20.5)**	**297.4 ± 5.63 (17)**	**294.4 ± 3.66 (10)**	**295.9 ± 4.29 (12.5)**	**295.8 ± 5.68 (18)**	**292.6 ± 3.96 (12)**	**294.2 ± 4.17 (12)**	**295.0 ± 5.50 (17)**	**294.6 ± 4.31 (14)**	**294.9 ± 4.48 (11.5)**

Table 5.6 Tear osmolarity values for **Normal** subjects following 45 minutes exposure to 70% RH

Subject	tOsm clinic conditions (mOsm/L)			tOsm on eye opening (mOsm/L)			tOsm 15 minutes (mOsm/L)			tOsm 30 minutes (mOsm/L)			tOsm 45 minutes (mOsm/L)		
	RE	LE	BE	RE	LE	BE	RE	LE	BE	RE	LE	BE	RE	LE	BE
1~	316	299	307.5	285	291	288	303	295	299	301	297	299	299	293	296
2~	302	304	303	282	283	282.5	316	310	313	293	*	*	295	304	299.5
3	295	306	300.5	283	287	285	286	302	294	293	298	295.5	294	301	297.5
4~	283	291	287	277	275	276	283	281	282	283	280	281.5	282	283	282.5
5~	297	284	290.5	283	284	283.5	286	305	295.5	296	293	294.5	287	297	292
6	303	301	302	290	286	288	296	287	291.5	298	290	294	305	303	304
7	298	307	302.5	282	285	283.5	291	290	290.5	286	292	289	300	295	297.5
8	306	326	316	288	298	293	302	301	301.5	312	304	308	308	303	305.5
Mean ± SD (range)	**301.3 ± 7.36 (23)**	**302.3 ± 12.40 (42)**	**301.1 ± 9.11 (29)**	**283.8 ± 3.99 (13)**	**286.1 ± 6.60 (23)**	**284.9 ± 4.98 (17)**	**295.4 ± 11.19 (33)**	**296.4 ± 9.86 (29)**	**295.9 ± 9.09 (31)**	**294.0 ± 9.56 (29)**	**295.3 ± 8.66 (28)**	**295.3 ± 7.88 (26.5)**	**296.3 ± 8.71 (26)**	**297.4 ± 7.09 (21)**	**296.8 ± 7.21 (23)**

~ Patients diagnosed with Sjögren syndrome

* Represents a discarded reading due to difficulty in sampling

Table 5.7 Tear osmolarity values for **DED** patients following 45 minutes of eye closure and 45 minutes exposure to 45% RH

Subject	tOsm clinic conditions (mOsm/L)			tOsm 15 minutes (mOsm/L)			tOsm 30 minutes (mOsm/L)			tOsm 45 minutes (mOsm/L)		
	RE	LE	BE	RE	LE	BE	RE	LE	BE	RE	LE	BE
1~	303	306	304.5	308	307	307.5	308	305	306.5	302	301	301.5
2~	303	302	302.5	272	300	286	345	337	341	310	329	319.5
3	294	297	295.5	288	295	291.5	290	299	294.5	293	288	290.5
4~	290	286	288	284	285	284.5	283	285	284	282	284	283
5~	290	291	290.5	289	290	289.5	290	295	292.5	295	293	294
6	308	302	305	306	301	303.5	307	305	306	302	289	295.5
7	297	301	299	*	289	*	*	288	*	*	285	*
8	291	306	298.5	288	295	291.5	287	290	288.5	288	291	289.5
Mean ± SD (range)	**297.9 ± 7.01 (18)**	**298.9 ± 7.14 (20)**	**297.8 ± 6.76 (17)**	**290.7 ± 12.55 (36)**	**295.3 ± 7.23 (23)**	**293.4 ± 8.73 (23)**	**301.4 ± 21.53 (62)**	**300.5 ± 16.53 (52)**	**301.9 ± 19.19 (57)**	**296.0 ± 9.47 (28)**	**295.0 ± 14.7 (45)**	**296.2 ± 11.75 (36.5)**

~ Patients diagnosed with Sjögren syndrome

* Represents a discarded reading due to difficulty in sampling

Table 5.8 Tear osmolarity values for **DED** patients following 45 minutes exposure to 70% RH

The effect of eye closure on tear osmolarity

In the normal group, there was a significant decrease in tOsm in both the RE and LE from that measured in clinic conditions immediately on eye opening, following 45 minutes of eye closure (RE $F_{(4,28)} = 6.839$ $p = 0.001$; LE $F_{(4,28)} = 3.591$ $p = 0.017$). The average clinic tOsm in the RE was 292.9 ± 2.91 mOsm/L and fell to 286.3 ± 2.71 mOsm/L ($p = 0.015$) on eye opening. The average clinic tOsm in the LE was 293.1 ± 5.54 mOsm/L, falling to 285.9 ± 5.16 mOsm/L ($p = 0.006$) on eye opening. The tOsm returned towards the clinic value in both the RE and LE eyes (RE $p = 0.50$; LE $p = 0.51$) in the period of exposure to 45% RH that followed.

In the DED group, there was a significant decrease in tOsm in the RE and LE from that measured in clinic conditions, to that obtained immediately on eye opening (RE $F_{(4,28)} = 10.421$ $p = 0.000$; LE $X^2_{(4)} = 17.139$ $p = 0.002$). The average clinic tOsm in the RE was 301.3 ± 7.36 mOsm/L, falling to 283.8 ± 3.99 mOsm/L ($p = 0.0002$). The average clinic tOsm in the LE was 302.3 ± 12.4 mOsm/L. falling to 286.1 ± 6.60 mOsm/L ($p = 0.01$). As with the normal group, tOsm returned towards the clinic value in both the RE and LE eyes (RE $p = 0.182$; LE $p = 1.0$) in the period of exposure to 45% RH that followed. This is presented graphically in Figures 5.2-5.7.

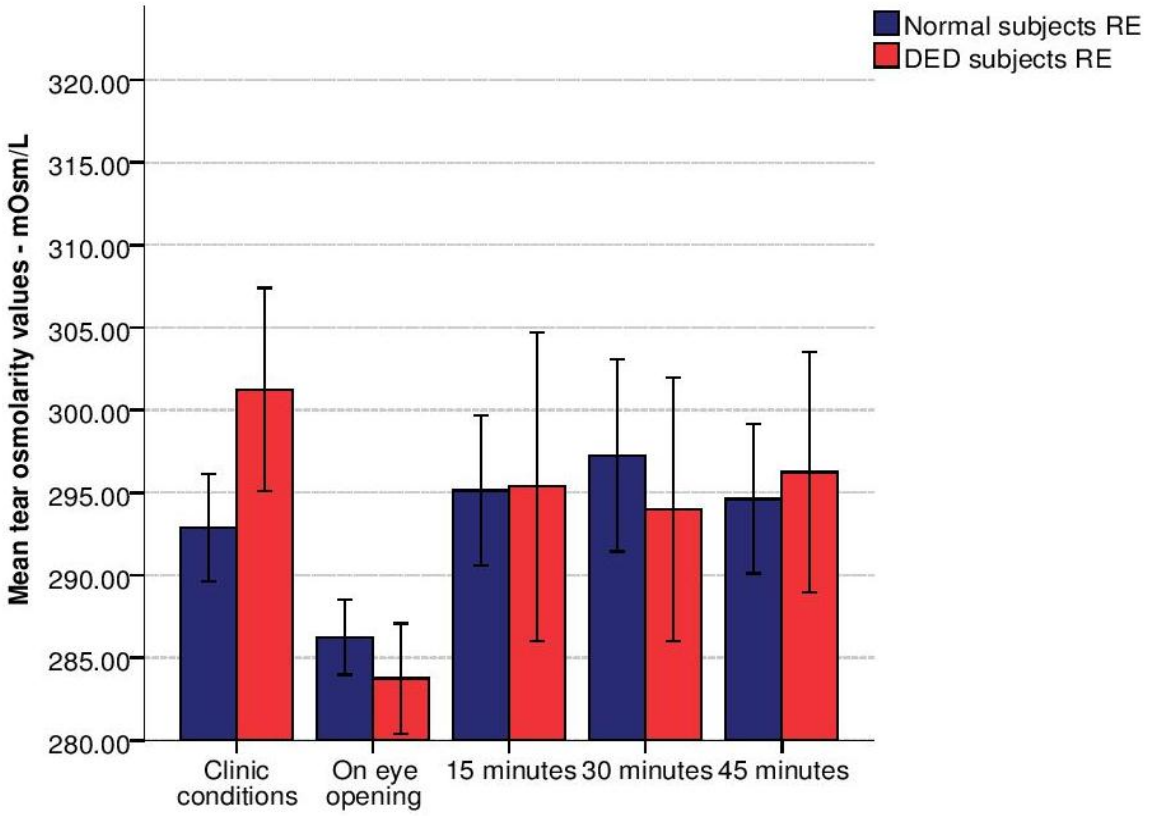


Figure 5.2 Bar graph displaying mean tOsm values for normal and DED subjects (right eye) after 45 minutes of eye closure and then every 15 minutes with eyes open (exposure to 45% RH).

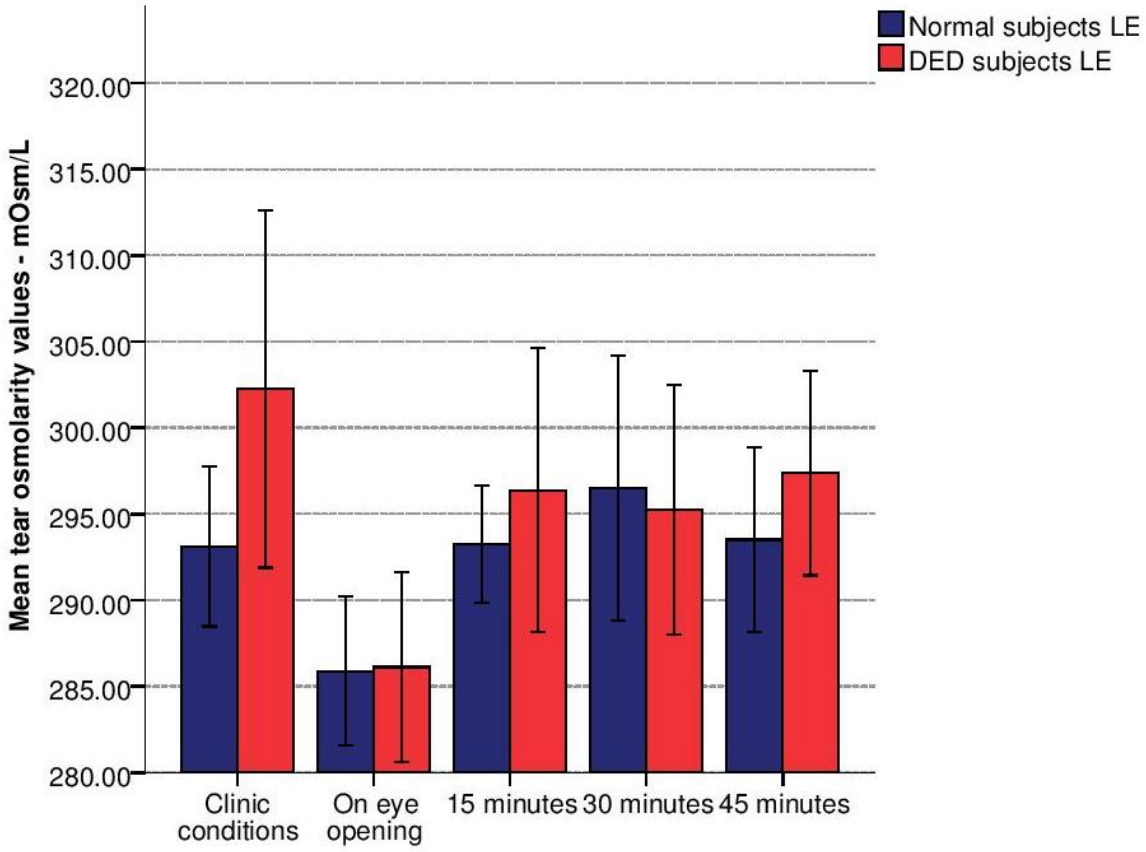


Figure 5.3 Bar graph displaying mean tOsm values for normal and DED subjects (left eye) after 45 minutes of eye closure and then every 15 minutes with eyes open (exposure to 45% RH).

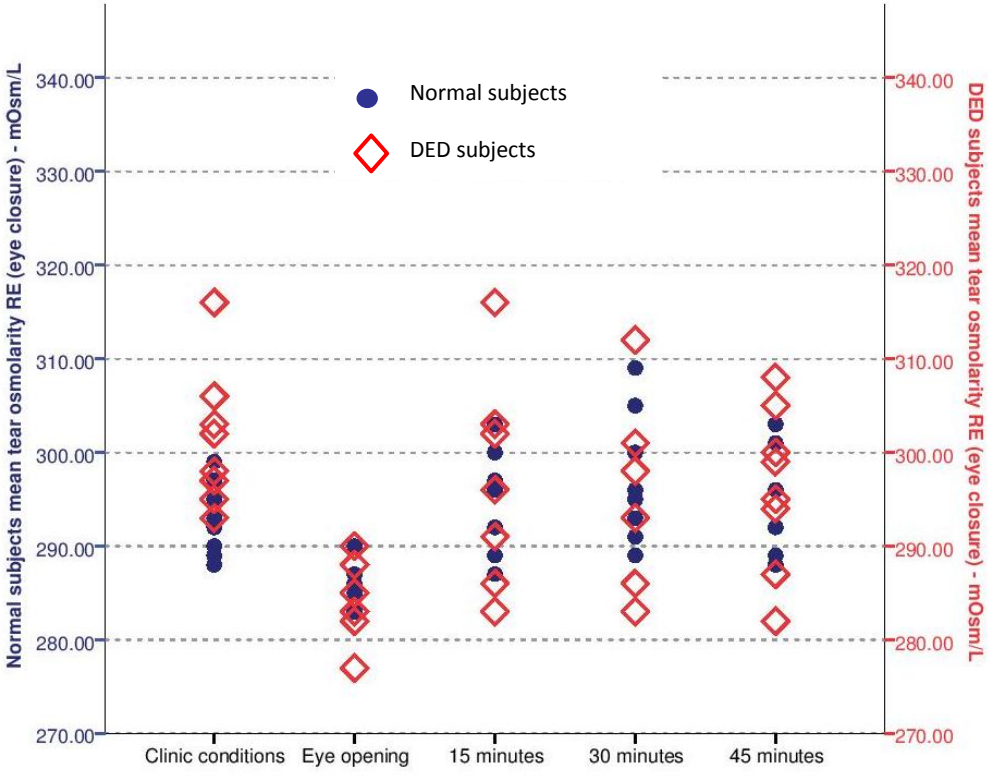


Figure 5.4 Scattergraph displaying mean tOsm values for normal and DED subjects (right eye) after 45 minutes of eye closure and then every 15 minutes with eyes open (exposure to 45% RH).

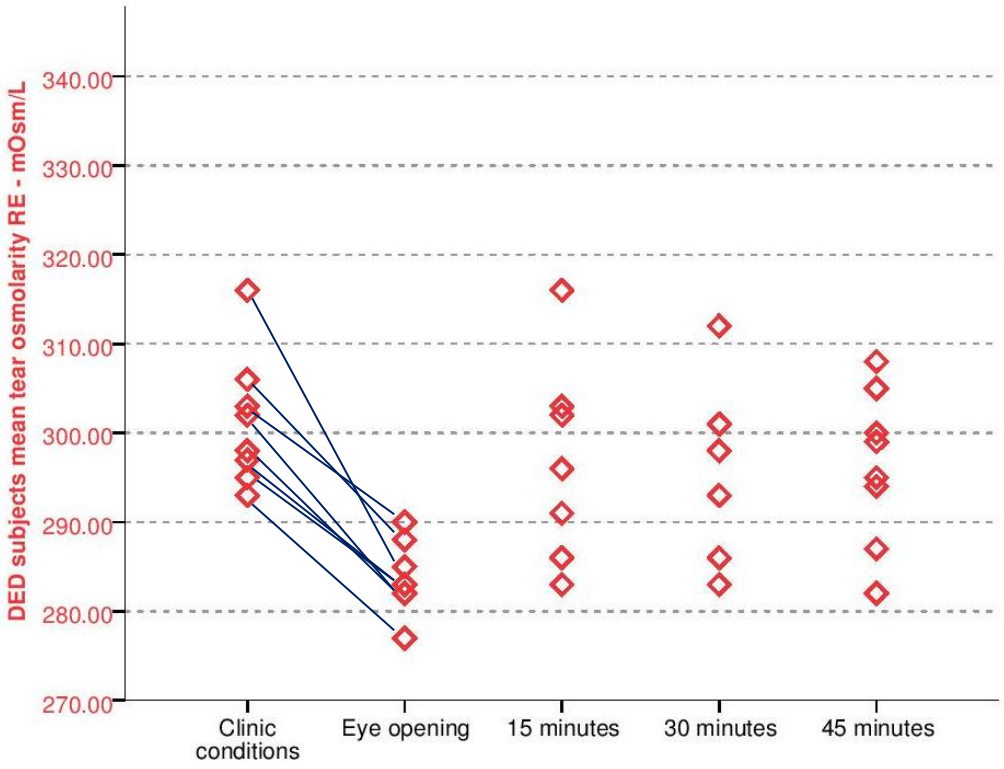


Figure 5.5 Scattergraph displaying mean tOsm values for DED patients (right eye) after 45 minutes of eye closure and then every 15 minutes with eyes open (exposure to 45% RH). Lines highlight the tOsm values in clinic conditions of individuals and the subsequent levels of tOsm achieved following eye closure.

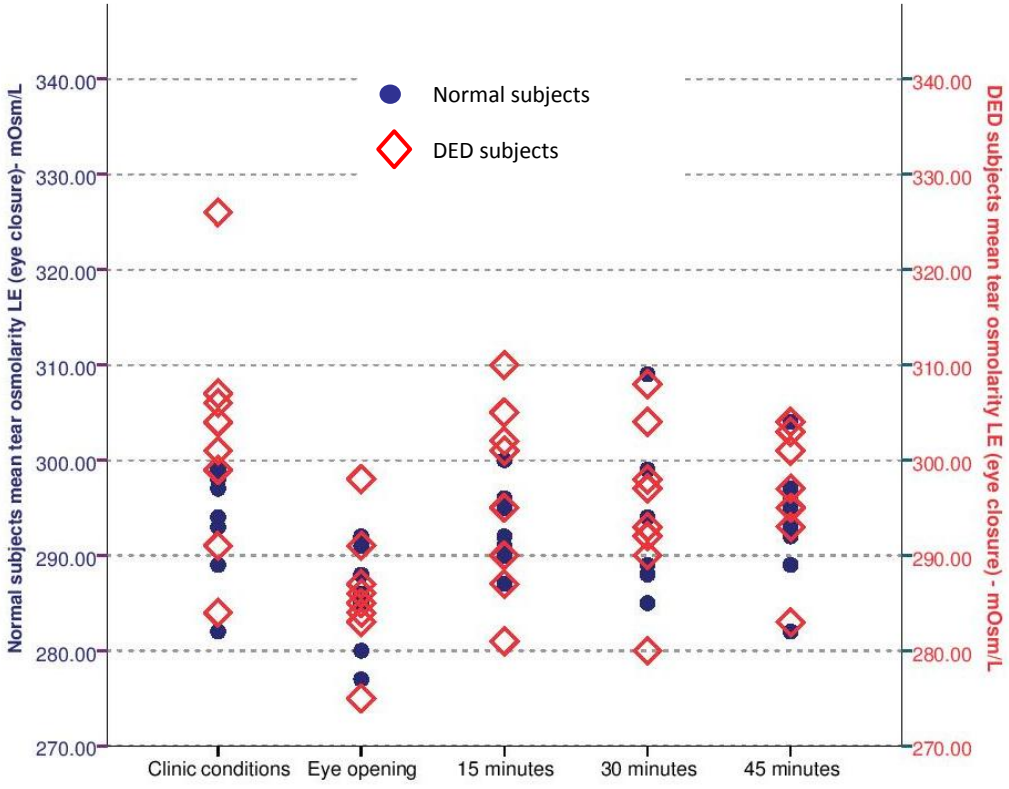


Figure 5.6 Scattergraph displaying mean tOsm values for normal and DED subjects (left eye) after 45 minutes of eye closure and then every 15 minutes with eyes open (exposure to 45% RH).

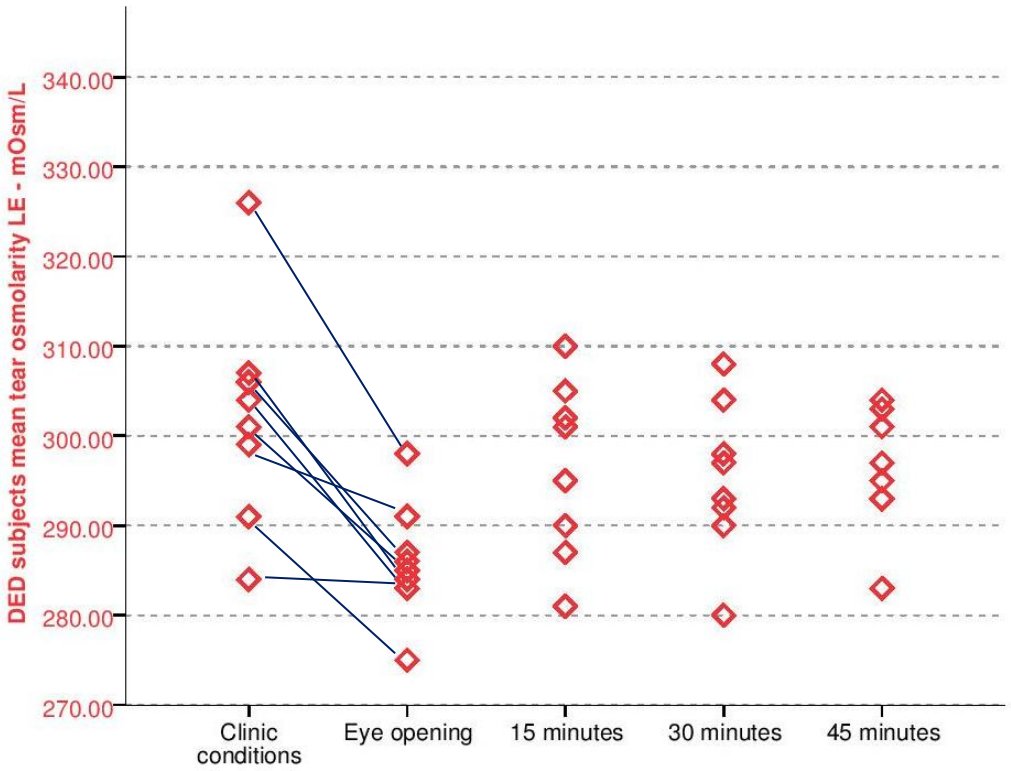


Figure 5.7 Scattergraph displaying mean tOsm values for DED patients (left eye) after 45 minutes of eye closure and then every 15 minutes with eyes open (exposure to 45% RH). Lines highlight the tOsm values in clinic conditions of individuals and the subsequent levels of tOsm achieved following eye closure.

The effect exposure to 70% relative humidity on tear osmolarity

In the normal group, there was a decrease in tOsm measured in the RE and LE from that measured in clinic conditions, to that obtained following 45 minutes exposure to 70% RH. This reached significance in the LE only (RE $F_{(1.241,8.686)} = 2.083$ $p = 0.133$; LE $F_{(1.235,8.646)} = 5.056$ $p = 0.047$). The average clinic tOsm in the RE was 298.8 ± 8.91 mOsm/L and fell to 295.0 ± 5.50 mOsm/L ($p = 1.108$). The average clinic value in the LE was 300.3 ± 7.48 mOsm/L, falling to 294.6 ± 4.31 mOsm/L ($p = 0.045$).

In the DED group, there was a decrease in tOsm measured in the RE and LE from clinic conditions, compared to that obtained following 45 minutes exposure to 70% RH, but neither eye reached significance (RE $X^2_{(3)} = 3.716$ $p = 0.294$; LE $X^2_{(4)} = 6.722$ $p = 0.081$). The RE started at 297.9 ± 7.01 mOsm/L and fell to 296.0 ± 9.47 mOsm/L ($p = 0.294$). The LE started at 298.9 ± 7.14 mOsm/L and fell to 295.0 ± 14.7 mOsm/L ($p = 0.081$). This is presented graphically in Figures 5.8-5.11.

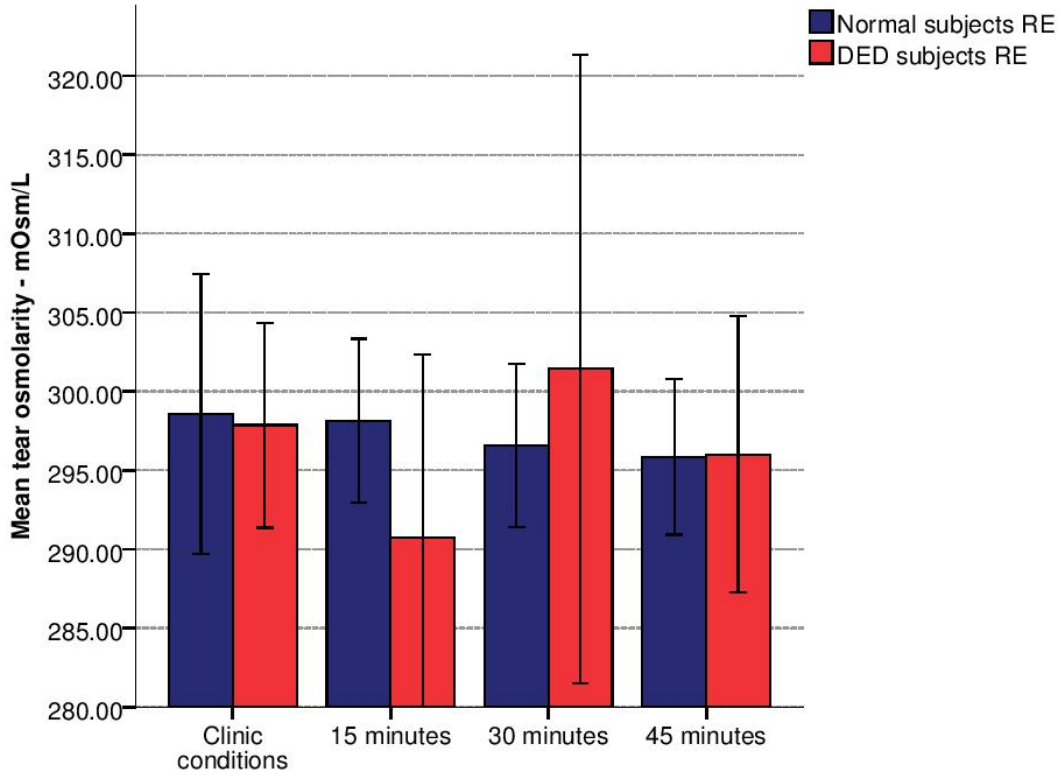


Figure 5.8 Bar graph displaying mean tOsm values for normal and DED subjects (right eye) every 15 minutes with eyes open (exposure to 70% RH).

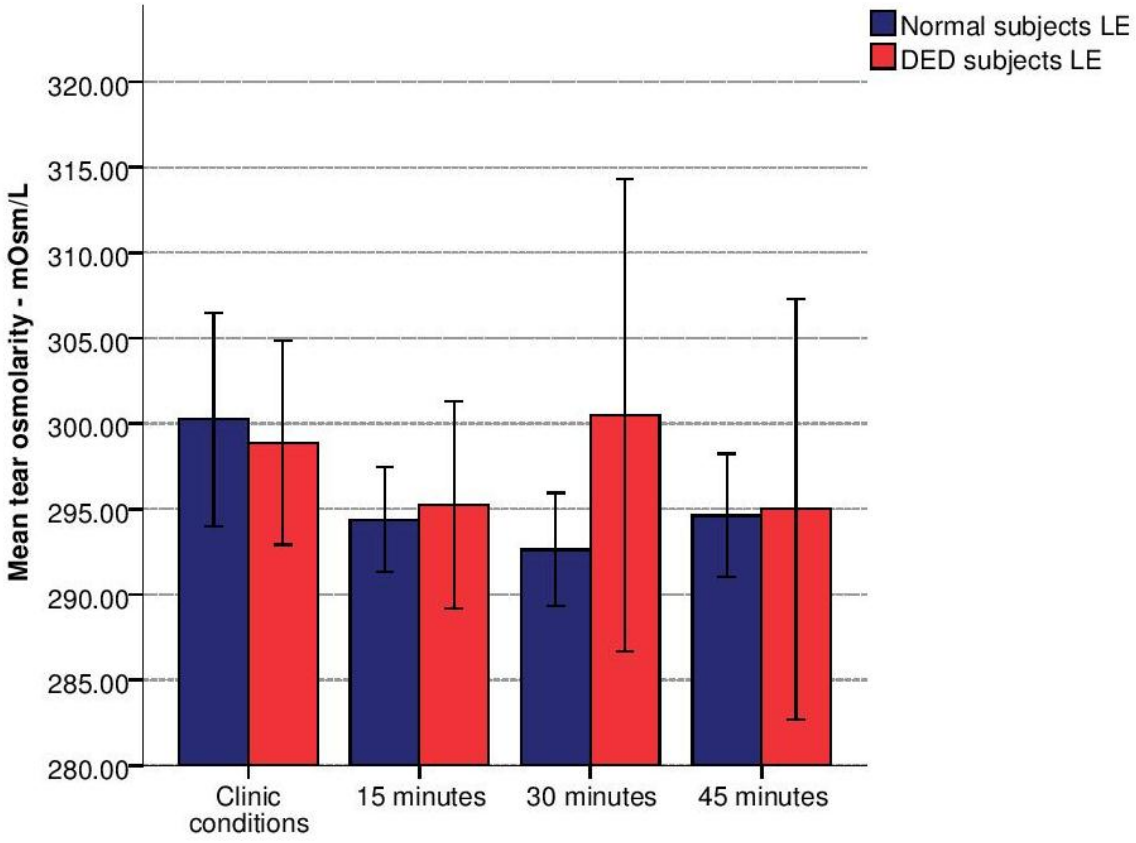


Figure 5.9 Bar graph displaying mean tOsm values for normal and DED subjects (left eye) every 15 minutes with eyes open (exposure to 70% RH).

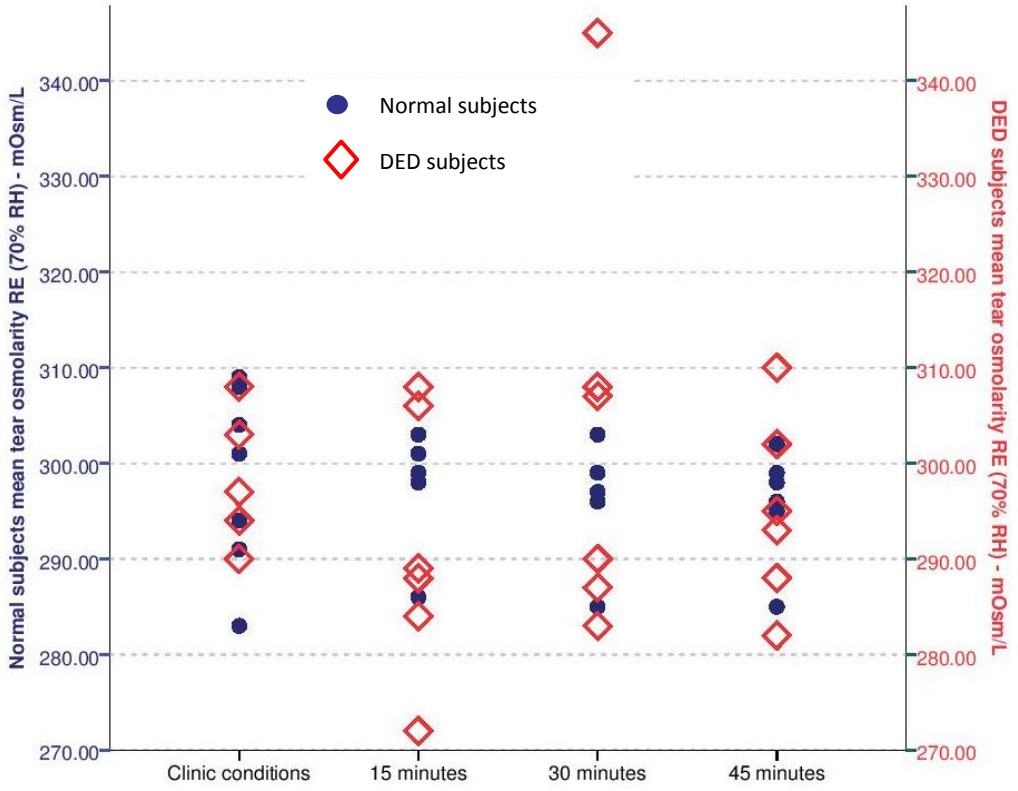


Figure 5.10 Scattergraph displaying mean tOsm values for normal and DED subjects (right eye) every 15 minutes with eyes open (exposure to 70% RH).

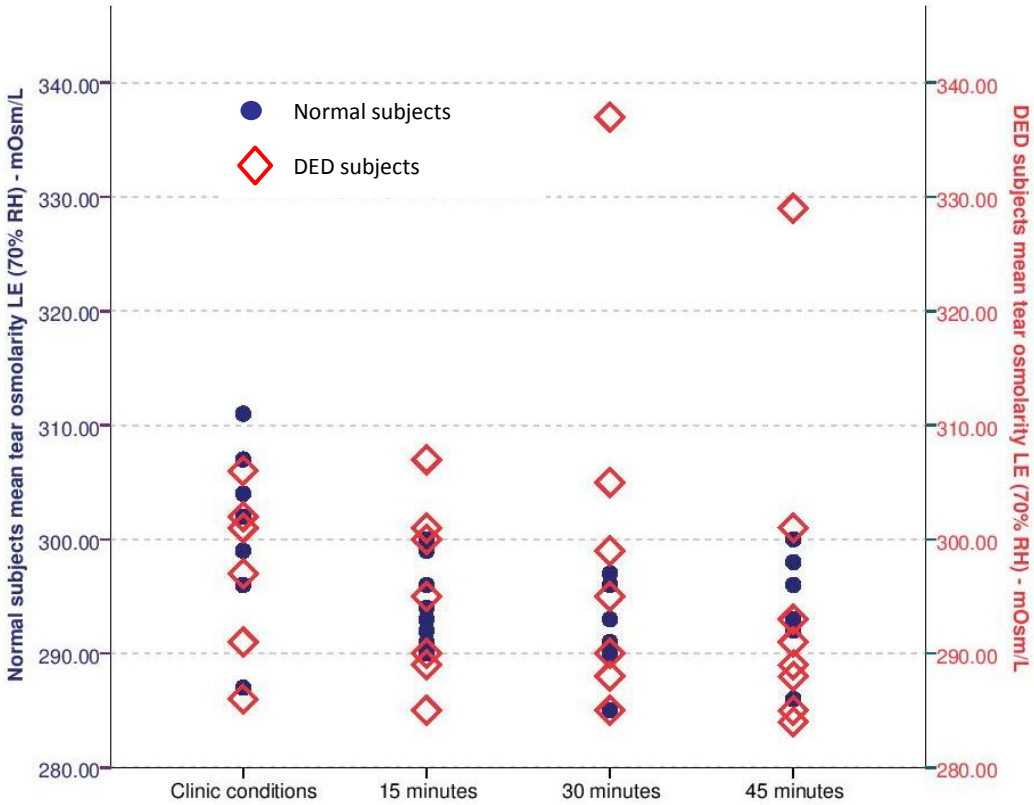


Figure 5.11 Scattergraph displaying mean tOsm values for normal and DED subjects (left eye) every 15 minutes with eyes open (exposure to 70% RH).

Effect of eye closure on tear osmolarity - averaged between right and left eyes

There was a significant decrease in tOsm, averaged between the two eyes from that measured in clinic conditions, to that obtained immediately on eye opening (Normal $F_{(2.23,15.608)} = 8.272$ $p = 0.003$; DED $F_{(4,28)} = 15.268$ $p = 0.000$). The average clinic value in the normal group fell from 293.0 ± 4.05 mOsm/L to 286.1 ± 3.57 mOsm/L ($p = 0.045$) and in the DED group, from 301.1 ± 9.11 mOsm/L to 284.9 ± 4.98 mOsm/L ($p = 0.000118$). The tOsm returned towards the clinic value in both the RE and LE eyes (normal $p = 1.06$; DED $p = 0.25$), in the period of exposure to 45% RH, that followed. This is presented graphically in Figures 5.12-5.14.

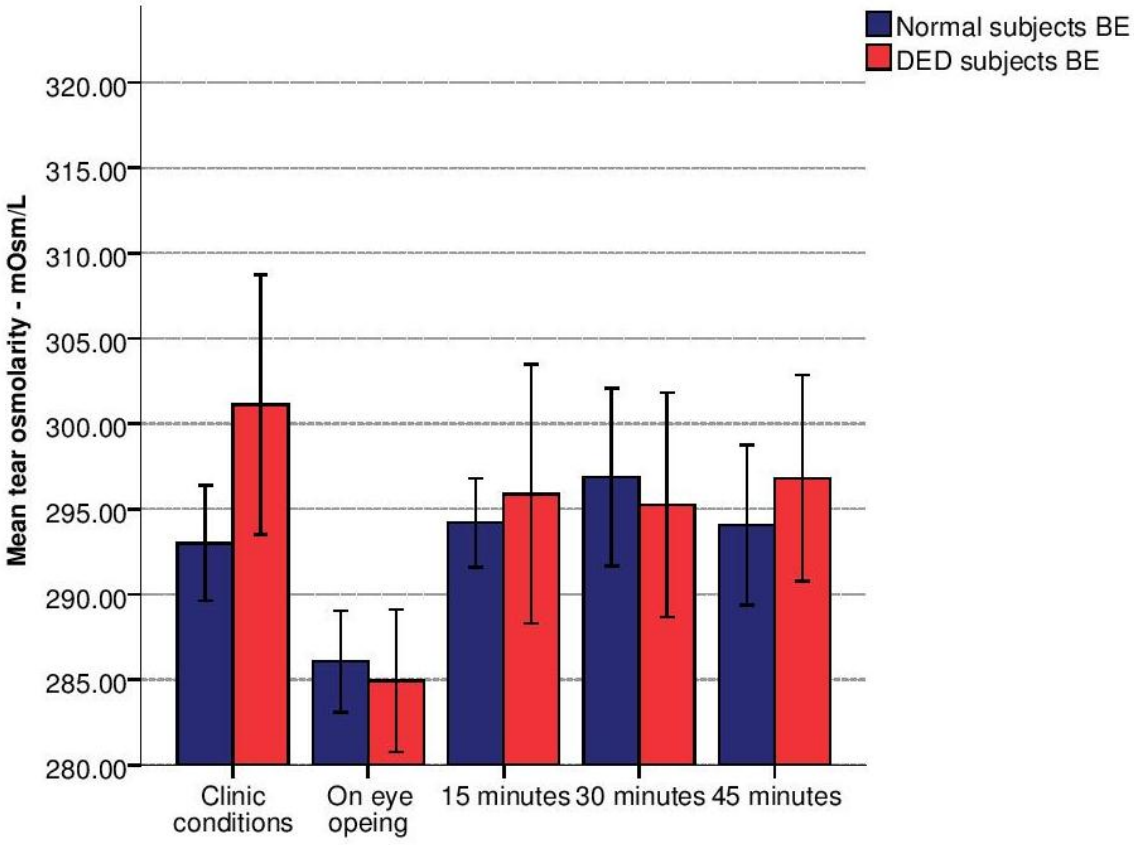


Figure 5.12 Bar graph displaying mean tOsm values for normal and DED (both eyes averaged) subjects after 45 minutes of eye closure and then every 15 minutes with eyes open (exposure to 45% RH).

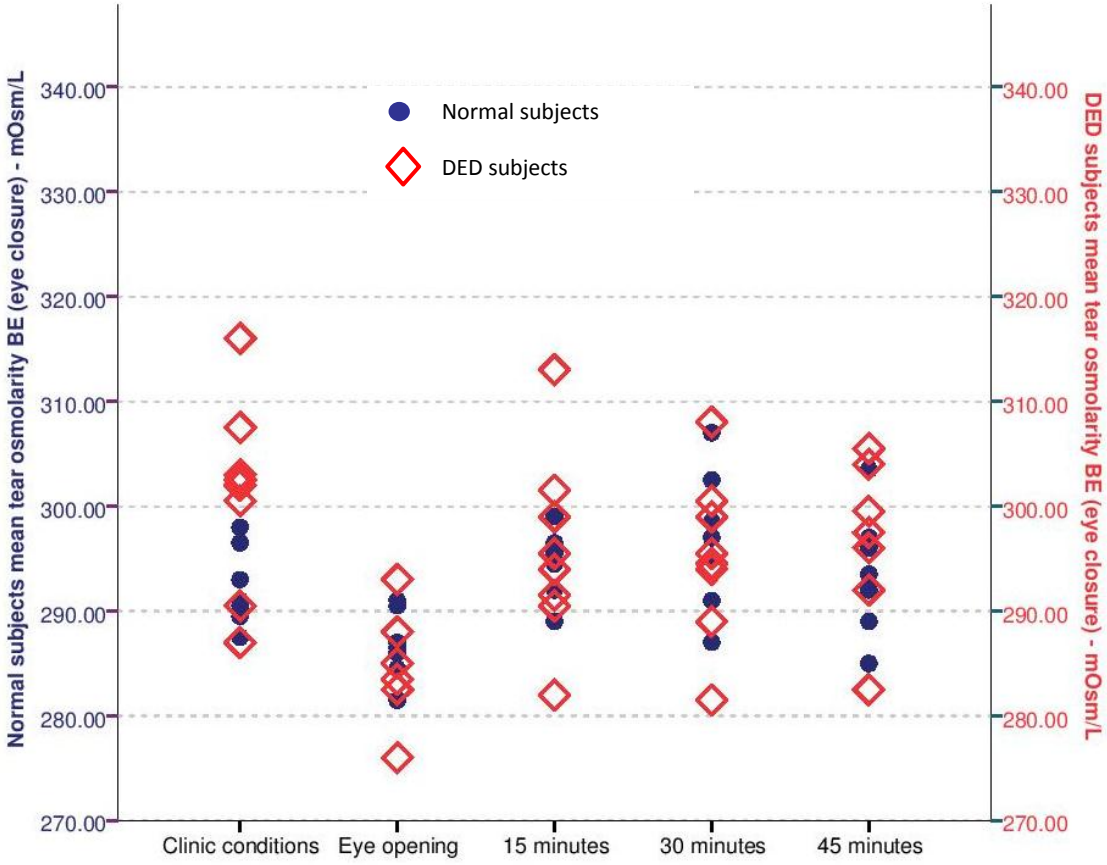


Figure 5.13 Scattergraph displaying mean tOsm values for normal and DED subjects (both eyes averaged) after 45 minutes of eye closure and then every 15 minutes with eyes open (exposure to 45% RH).

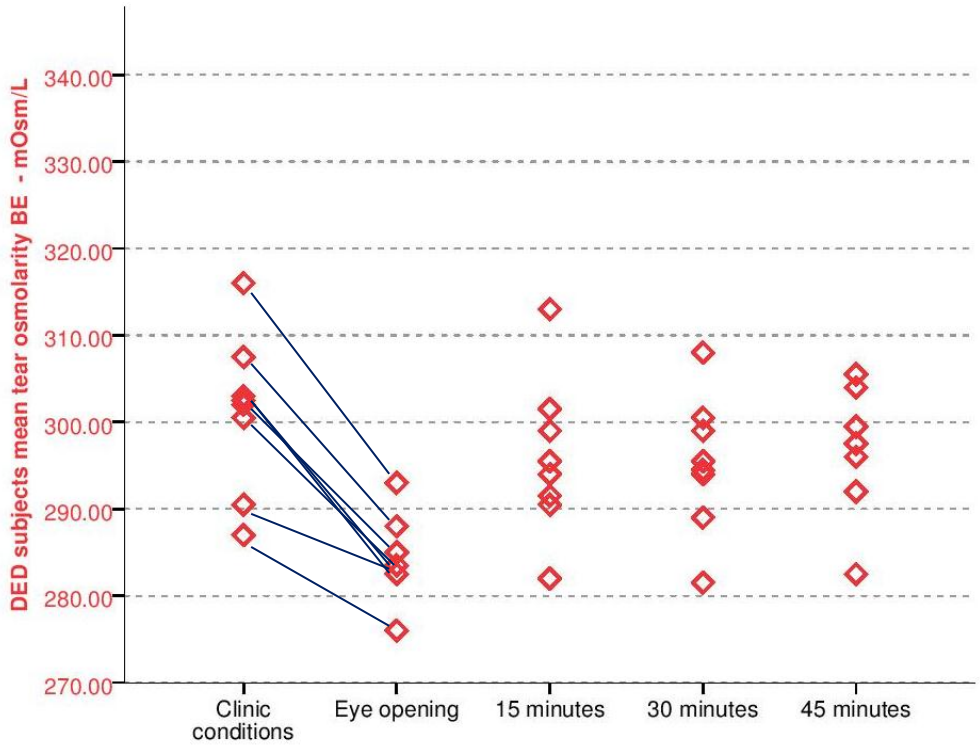


Figure 5.14 Scattergraph displaying mean tOsm values for DED patients (both eyes) after 45 minutes of eye closure and then every 15 minutes with eyes open (exposure to 45% RH). Lines highlight the tOsm values in clinic conditions of individuals and the subsequent levels of tOsm achieved following eye closure.

Effect of 70% relative humidity – averaged between right and left eyes

In the normal and DED, group there was a decrease in tOsm, averaged between the two eyes from that measured in clinic conditions, compared to that obtained following 45 minutes exposure to 70% RH however, this did not reach significance (Normal $F_{(1.174,8.216)}$ = 3.553 p = 0.92; DED $X^2_{(3)}$ = 3.514 p = 0.319). The average clinic value in the normal group fell from 299.5 ± 7.78 mOsm/L to 294.9 ± 4.48 mOsm/L (p = 0.92), and in the DED group from 297.8 ± 6.76 mOsm/L to 296.2 ± 11.75 mOsm/L (p = 0.319). This is presented graphically in Figures 5.15-5.16.

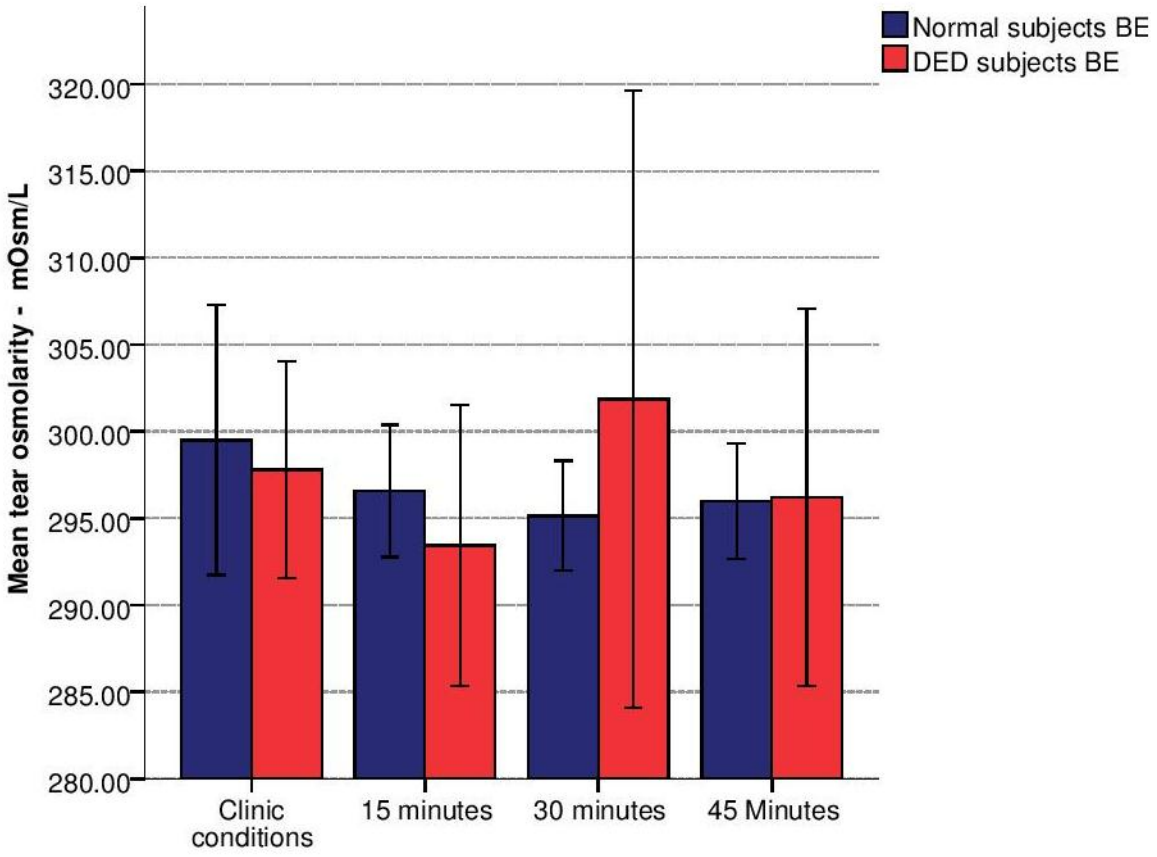


Figure 5.15 Bar graph displaying mean tOsm values for normal and DED subjects (both eyes averaged) every 15 minutes with eyes open (exposure to 70% RH).

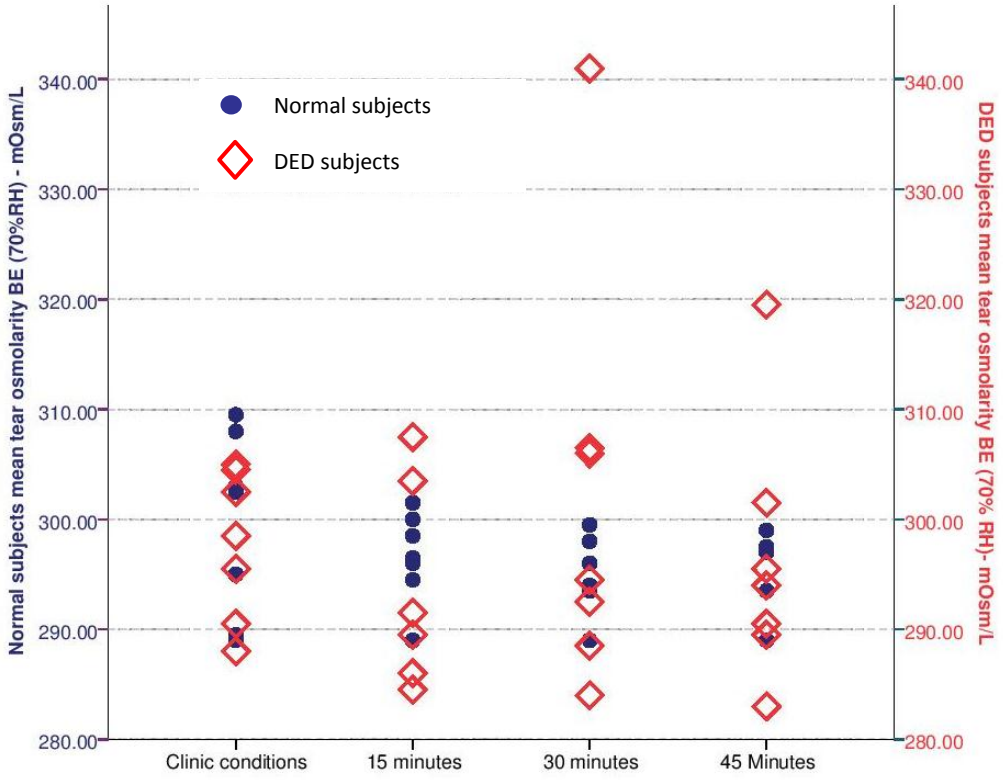


Figure5.16 Scattergraph displaying mean tOsm values for normal and DED subjects (both eyes averaged) every 15 minutes with eyes open (exposure to 70% RH).

Comparison of the effect eye closure exposure to 70% relative humidity on tear osmolarity

Normal subjects

The tOsm measured on eye opening after 45 minutes of eye closure was significantly lower than that obtained after 45 minutes exposure to 70% RH in the RE ($t_{(7)}$ = -4.129 p = 0.004) and LE ($t_{(7)}$ = -3.055 p = 0.018).

DED patients

The tOsm measured on eye opening was lower than that obtained after 45 minutes exposure to 70% RH; however, only the RE reached significance (RE Z = 2.268 p = 0.016; LE Z = 1.863 p = 0.063). This is presented graphically in Figures 5.17 and 5.18.

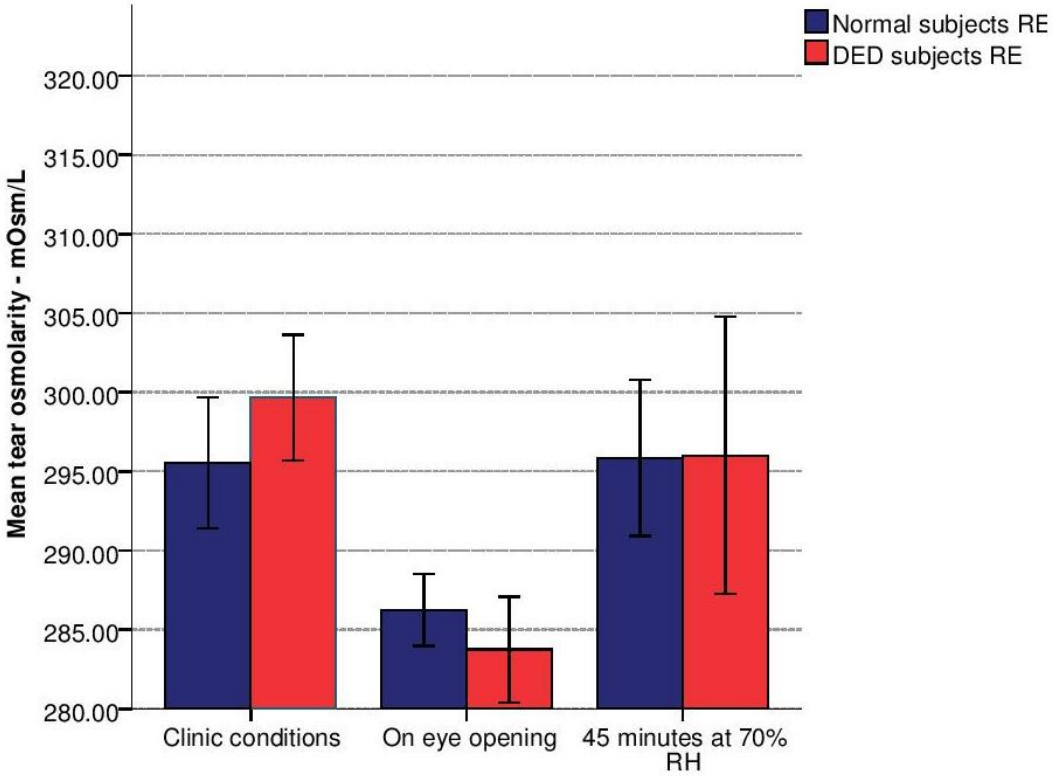


Figure 5.17 Bar graph displaying mean tOsm values for normal and DED subjects (right eye) in clinic conditions, after 45 minutes exposure to 70% RH and immediately after eye opening.

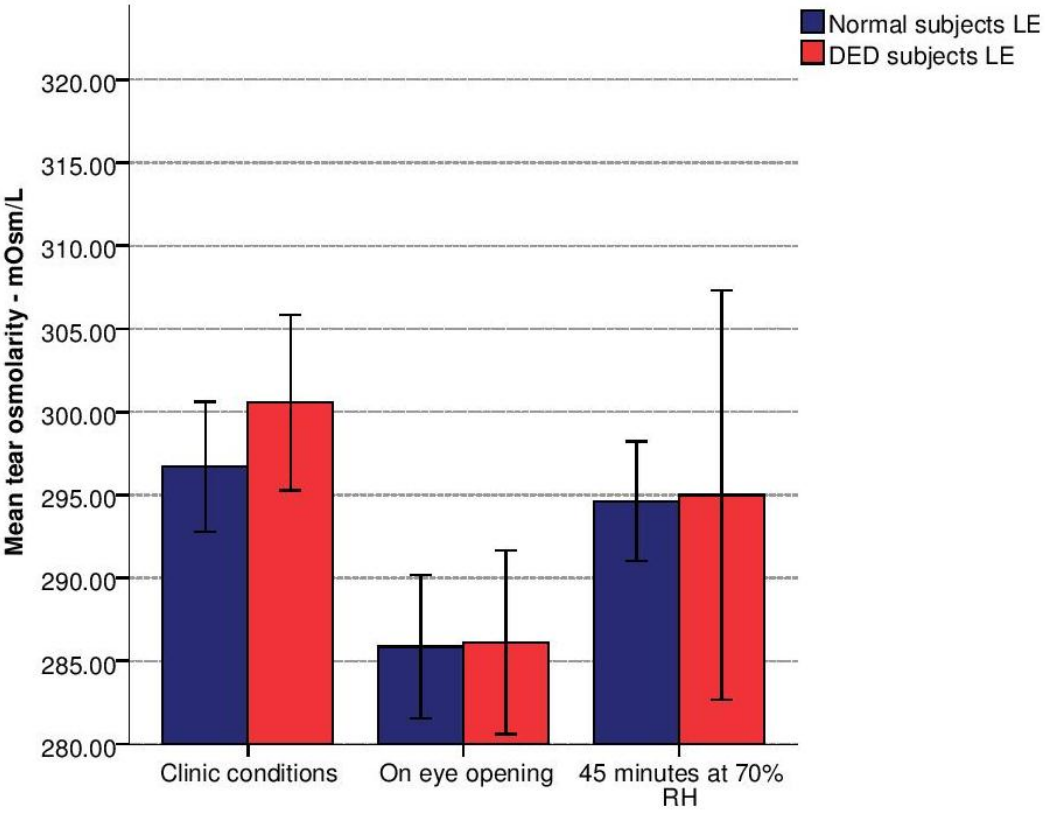


Figure 5.18 Bar graph displaying mean tOsm values for normal and DED subjects (left eye) in clinic conditions, after 45 minutes exposure to 70% RH and immediately after eye opening.

Comparison of the effect of eye closure and exposure to 70% relative humidity on tear osmolarity – averaged between right and left eyes

The averaged tOsm measured on eye opening was significantly lower than that obtained after 45 minutes exposure to 70% RH in both the normal group ($t_{(7)}$ = -3.699 p = 0.008) and the DED group (Z = 2.68 p = 0.016). This is presented graphically in figures 5.19.

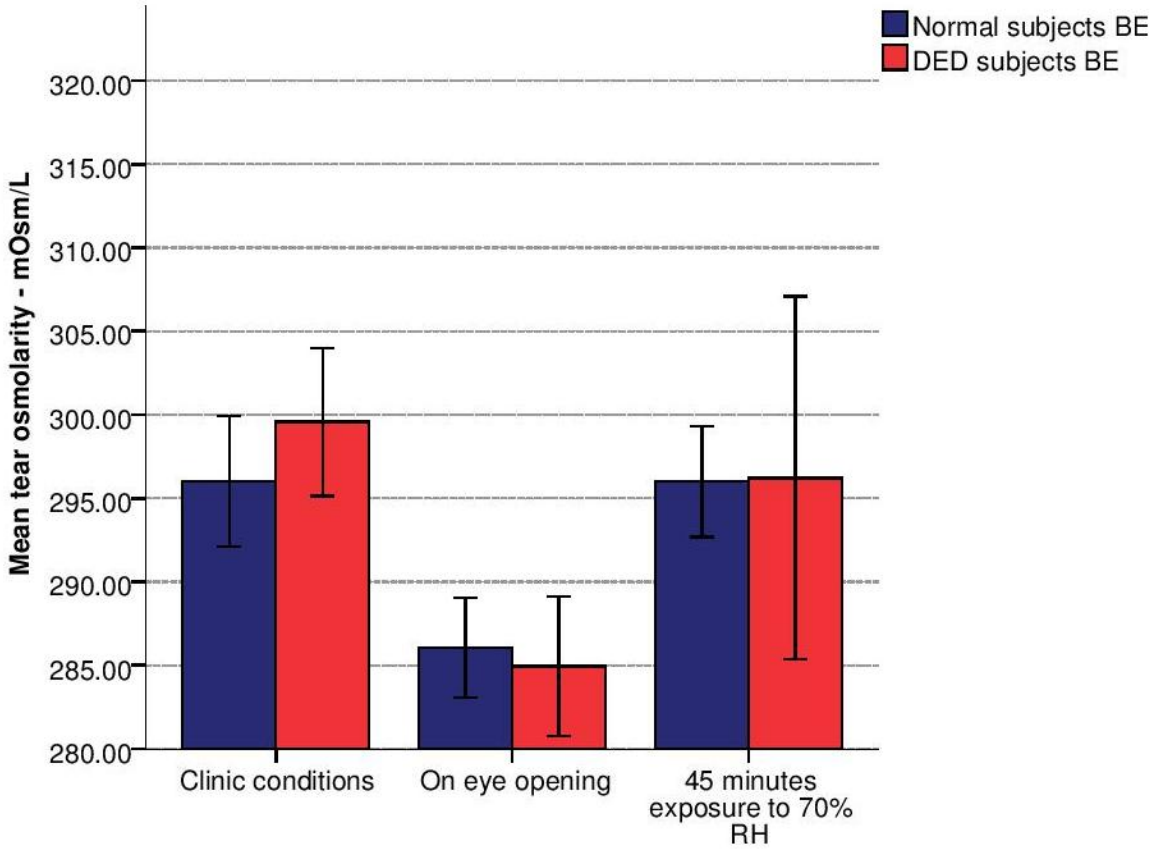


Figure 5.19 Bar graph displaying mean tOsm values for normal and DED subjects (both eyes averaged) in clinic conditions, after 45 minutes exposure to 70% RH and immediately after eye opening.

Inter-eye differences

The tOsm in clinic conditions was compared in both groups, with that following 45 minutes of eye closure and following 45 minutes exposure to 70% RH. A significant inter-eye difference was found in the DED group in clinic conditions: RE 295.9 ± 8.17 mOsm/L and LE 301.6 ± 10.63 mOsm/L (p = 0.017); but not in the normal group: RE 296.9 ± 6.86 mOsm/L and LE 297.8 ± 6.66 mOsm/L (p = 0.268). Significance was not demonstrated in inter-eye tOsm in either group after eye closure: normal group: RE 286.3 ± 2.93 mOsm/L and LE 287.1 ± 4.02 mOsm/L (p = 0.383); DED group: RE 285.5 ± 6.12 mOsm/L and LE 286.1 ± 6.60 mOsm/L (p = 0.809), or exposure to 70% RH: normal group: RE 295.3 ± 2.93 mOsm/L and LE 287.1 ± 4.02 mOsm/L (p = 0.383); DED group: RE 295.3 ± 10.58 mOsm/L and LE 291.0 ± 5.76 mOsm/L (p = 0.170).

No adverse events were recorded on slit-lamp examination following any of the experiments.

5.5 DISCUSSION

The current literature indicates that in normal human eyes, tear osmolarity (tOsm) measured in uncontrolled environmental conditions is higher than that of the secreted tears (Rolando and Refojo, 1983; Tsubota and Yamada, 1992; Mathers, 2004; Saleh et al., 2012). This effect is amplified when the eyes are exposed to ambient conditions of desiccating stress, such as low humidity, high temperature and high airflow (Gonzalez-Garcia et al., 2007; Madden, Tomlinson and Simmons, 2013; Teson et al., 2013; Peng et al., 2014; López-Miguel et al., 2014), and probably accounts for the fluctuations of tOsm over the course of the day (Farris, Stuchell and Mandel, 1986; Sullivan et al., 2012). Modelling considerations have further predicted that the tOsm of the preocular tear film is normally slightly higher than that of the tear meniscus and that this discrepancy is amplified in dry eye (Gaffney et al., 2010). In the current study it is assumed that this difference is abolished by the period of evaporative suppression, and is therefore absent at the time of sampling, so that the meniscus tOsm represents that of the tear fluid as a whole. In those studies where tOsm has been measured immediately, or shortly after, a prolonged period of eye closure, as in sleep, tears have been reported to be iso- or hypo-osmolar, with respect to plasma (Terry and Hill, 1978; Niimi et al., 2013). This is interpreted here as elsewhere, as a response to the total suppression of evaporation, but also to an equilibration of the tears during eye closure, with the vascular circulation across the conjunctival epithelium. It is assumed that this value of tOsm represents the lowest achievable at the surface of the eye and for this reason it is referred to here as the Basal Tear Osmolarity (BTO) although this concept will require further experimentation to validate.

The present study supports the prediction that evaporative suppression can drive down tOsm. It was found that following a period of 45 minutes of eye closure, tOsm measured in the tear meniscus was significantly reduced in each eye, from that measured in uncontrolled clinic conditions in both the normal and DED groups. A smaller fall was recorded after exposure to 70% RH in open-eye, but only reached significance in the LE of the normal group (p = 0.045). It is acknowledged that in this study the male to female ratio in the DED group (1M:7F) differed from that in the normal group (4M : 4F) and the mean age of the DED group was greater. However, there was no expectation that this would influence the overall results.

In the closed eye study in the normal group, tOsm fell significantly in the RE, from an average clinic value of 292.9 ± 2.91 mOsm/L to 286.3 ± 2.71 mOsm/L (p = 0.015), and in the LE from an average clinic level of 293.1 ± 5.54 mOsm/L, to 285.9 ± 5.16 mOsm/L (p = 0.006) on eye opening. In the DED group there was a significant decrease in tOsm in the RE from 301.3 ± 7.36 mOsm/L to 283.8 ± 3.99 mOsm/L (p = 0.0002), and in the LE from 302.3 ± 12.4 mOsm/L to 286.1 ± 6.60 mOsm/L (p = 0.01). Importantly, all tOsm values recorded on eye opening fall within the range accepted for plasma osmolality, 285-295 mOsm/Kg (Cheuvront et al., 2010b; Matz, 1996; Stookey, 2005), which supports the hypothesis that complete evaporative suppression, for the period selected here, is capable of driving down tOsm to a value which is both basal, and close to that of pOsm, in both normal and DED patients. It is noted here that although the DED patients studied here at the time of recruitment had a tOsm of ≥308 mOsm/L, in the event, at the time of the experiments, their clinic tOsm values, except in the case of two subjects, were within the normal range. This may reflect the day-to-day variation of tOsm that occurs in DED. Additionally, seven of the DED patients were actively using a topical artificial tear preparation containing hyaluronate, which has been reported, after long-term use, to cause a prolonged fall in tOsm (Montani, 2013). This may be relevant here, despite the fact that all subjects were instructed to cease drop instillation for at least 4 hours prior to their assessments.

The 70% RH level employed in the present study was at the time the maximum achievable by the controlled environment chamber (CEC) used in the study, but although Madden et al., (2013) had concluded that tear evaporation was almost completely suppressed at this level of humidity (Madden, Tomlinson and Simmons, 2013), it is apparent from the current results that 70% RH did not achieve the complete suppression produced by eye closure. It is likely that this fact along with the small subject numbers recruited into the study are the reasons for a lack of significance found in this evaporative suppression condition for the majority of the experimental groups.

The two approaches selected here to achieve evaporative suppression, lid closure and exposure to high humidity would not be expected to give identical results, even had it been possible to expose eyes to 100% humidity. For one thing, the exposure times differ; the initial 10 minutes of the humidity experiment are needed for chamber equilibration (the time taken for the CEC to reach the specified conditions after subject entry), so that the period of exposure to high humidity is slightly curtailed. Despite this, it may be expected that the fall in osmolarity towards the BTO would be faster in the open-eye state than during eye closure, because tear mixing will be encouraged by spontaneous blinking and tear flow is likely to be higher in the absence of eye closure. It is also worth noting that in some people during eye closure, and in the absence of lid pathology, it has been shown that there is not always contact between the upper and lower lid margins, leading to a

potential failure of the eyelids to form an adequate moisture seal and protect against a degree of evaporation. Blackie and Korb (2015) used a halogen fibre optic transilluminator placed on the closed upper eyelid of healthy adults (n=116) to detect visible light emanation from between the lid margins (Blackie and Korb, 2015). Thus, even in a closed eye state some people may still experience a level of evaporation from the ocular surface.

For dry eye diagnosis it has been recommended that tOsm is measured bilaterally, since for instance, the inter-eye difference is increased in DED (Lemp et al., 2011). The current study as predicted, demonstrated less inter-eye variation in tOsm after eye closure and exposure to 70% RH in the DED group (eye closure p = 0.809; 70% RH p = 0.170), than that shown in uncontrolled, clinic conditions (p = 0.0017). A significant difference in inter-eye variability was not demonstrated in the normal group in uncontrolled clinic conditions (p = 0.268), or after complete evaporative suppression (eye closure p = 0.383; 70% RH p = 0.70).

Tear osmolarity in open eye conditions has been proposed by Fortes et al., (2011) as a non-invasive surrogate for plasma osmolality, of potential use in the rapid detection of dehydration in the elderly (Fortes et al., 2011). In the present study it has been predicted that complete evaporative suppression for a suitable period of time will drive down tOsm to a basal level that will more accurately reflect that of the pOsm. Although this can only be established by a direct comparison of the BTO with pOsm, the current results are encouraging. The tOsm measured following eye closure in the normal groups was 286.3 ± 2.71in the RE and 285.9 ± 5.16 in the LE, and in the DED group 283.8 ± 3.99 in the RE and 286.1 ± 6.60 in the LE. After 45 minutes exposure to 70% RH the tOsm in the normal group was 295.0 ± 5.5 in the RE and 294.6 ± 4.31 in the LE, and in the DED group 296.0 ± 9.47 in the RE and 295.0 ± 14.7 in the LE.

The results from this study have implications for the use of tOsm measurements as a means to determine body hydration. It is contended that the measurement of tOsm after a period of evaporative suppression will provide an osmolarity value that is closer to pOsm, is personal to the individual, and has a lower variance than that measured in open eye, uncontrolled environmental conditions. With the approach described here, a raised tOsm measured in open eye conditions can be taken at face value and interpreted, in conjunction with other clinical features, as a manifestation of DED. If this measurement is then followed with that of the tOsm after a period of lid closure, to obtain the BTO, then this tOsm value can be interpreted in terms of body hydration. The quick and easy measure of tOsm could be of use in rapid detection of dehydration, particularly in the Care Home setting (Wolff, Stuckler and McKee, 2015).

The results presented here support the view that in DED patients, as in normals, tOsm will be driven down to a basal level during the hours of sleep. Based on this research, the tOsm obtained following evaporative suppression could also provide a method of gauging DED severity. A comparison of an individual's presenting tOsm with their BTO, which is believed to represent the lowest tOsm value achievable in that individual could be calculated. The larger the interval between the two values would represent a greater degree of severity of the condition.

In terms of achieving a measure of the BTO, there is a practical value in adopting lid closure for the purposes of a clinical test, since it does not require specialist equipment such as a CEC or goggles to create a humid environment. The original selection of 45 minutes for eye closure was initially chosen as an arbitrary timescale, however this does fit within with the modelling consideration of Zhu and Chauhan (2007) who predicted that on returning to a normal level of evaporation after exposure to four times that rate, tOsm was found to return to baseline within 13 minutes (Zhu and Chauhan, 2007). Based on further calculations, it is anticipated that the time for eye closure in a practical test might be as low as 15 minutes (Willshire et al., 2018).

It will also be important to extend these studies, including measurement of the BTO at 95% RH, which should allow tracking of the reduction in tOsm over time, during exposure, since meniscus sampling can be conducted at any point throughout the exposure period. This would also serve to better define the period of eye closure required to devise a clinical test. If it transpires from future studies that the BTO can be acquired after a short period of eye closure, say 15 minutes or less, regardless of the starting level of tOsm, then the utility of the test will be greatly enhanced, e.g. for sports hydration medicine. This approach was recently investigated by Ungaro et al., (2015) who compared mean tOsm (averaged between right and left eyes), with pOsm in a group of male athletes before and after exercise tasks conducted on a stationary cycle ergometer (Ungaro et al., 2015). These tasks were carried out under controlled environmental conditions, with or without water restriction leading to up to 3% of body mass loss, and also after rehydration. They found that tOsm tracked group changes in hydration status similar to pOsm, but that individual responses of tOsm were less predictable. They concluded that tOsm is a valid indicator of hydration status at the group level, but that large differences among subjects in the response of tOsm to changes in hydration status limited its validity at the individual level. A similar conclusion was drawn in another study conducted under field conditions, involving a self-paced 10 km run in which participants were exposed to varied conditions of temperature, humidity and wind speed (Holland et al., 2017). In that study, although significant reductions in body mass and increases in pOsm, tOsm and urine specific gravity were observed, the pre- to post-exercise change in tOsm was not significantly correlated with pOsm, relative body mass loss, or urine osmolality or specific gravity. It

may be surmised that exclusion of environmental exposure, as proposed for a closed eye BTO test, might have revealed a correlation between tOsm and pOsm in such studies. The time taken to achieve the BTO value in a closed eye test will be important in determining its practicality, particularly under field conditions.

It is also important to observe the recovery of tOsm to pre-suppression levels on return to uncontrolled clinic conditions or, as performed here, to specified environmental conditions. The latter is of particular relevance to the real-life situation in DED where it may be supposed that tOsm is suppressed by overnight eye closure and climbs back to a condition of tear hyperosmolarity over the course of the day. The closed eye test allows this response to be observed closely, whether the eyes are returned to conditions of a standard office environment or to those of desiccating stress. The study by Niimi et al., (2013) demonstrated following a period of sleep, on eye opening the tOsm elevated to within those levels measured pre-sleep within 40 minutes (p = 0.085) (Niimi et al., 2013). This restoration phase may be predicted to differ between the major subtypes of DED, with a faster rise in EDE, in which the barrier to evaporative loss is compromised. It will be necessary to perform repeatability experiments in larger groups of subjects showing a wide range of tOsm, and also to perform additional studies comparing the BTO with the pOsm to strengthen conclusions.

To conclude, this is the first study to demonstrate that the tOsm of the tear film can be driven down to a lower level (BTO) employing the technique of eye closure. It seems that a period of 45 minutes of eye closure, or possibly less, should be sufficient to create a basal level of tOsm at the surface of the eye, although this conclusion requires confirmation in a larger group of subjects, including DED patients showing a greater degree of tear hyperosmolarity. It also gives confidence in the provisional conclusion that these values may not only represent a basal value, but that this value is close to and presumably determined in large part by plasma / extracellular fluid osmolarity levels. The BTO value achieved after eye closure promises to provide a personal norm against which the severity of DED in an individual and its response to treatment may be judged. It may also offer the opportunity to detect water-loss dehydration in the elderly using a simple bedside test.

The material presented here is reported in the publications:

Willshire, C., Buckley, R.J. and Bron, A.J., 2017b. Estimating basal tear osmolarity in normal and dry eye subjects. *Contact Lens and Anterior Eye*. [online] Available at: http://linkinghub.elsevier.com/retrieve/pii/S1367048417301790.doi: 10.1016/j.clae.2017.09.005

Willshire, C., Bron, A., Gaffney, E. and Pearce, E.I., 2018. Basal Tear Osmolarity as a metric to estimate body hydration and dry eye severity. *Progress in Retinal and Eye Research*. doi.org/10.1016/j.preteyeres.2018.02.001

Copies of these articles are contained in Appendix XIV.

Chapter 6:

CENTRAL CONNECTIONS OF THE LACRIMAL FUNCTIONAL UNIT

This study was designed to explore the relative contributions of each eye to the reflex tear response, measured by the Schirmer test in standard room conditions, after unilateral and bilateral topical anaesthetic blockade. The concept referred to here as 'cross-connectivity' relates to whether the afferent inputs from one cornea are processed in the ipsilateral superior salivatory nucleus (SSN) only, or have some input to the contralateral side also. This concept is relevant to many conditions that result in unilateral ocular anaesthesia such as neurotrophic keratitis (NK) and refractive surgery, where not only the affected eye may be compromised but also the fellow eye. Conversely, the unaffected fellow eye may be capable of a compensatory response that offsets the lack of sensory feedback from the affected eye.

6.1 INTRODUCTION

The tear film is constantly replenished by aqueous secretion from the lacrimal glands (main, palpebral and accessory), and is regularly refreshed by blinking and to a small extent by eye movements. The conjunctival epithelium makes a small contribution to aqueous tears. Secretion is finely adjusted in response to environmental conditions by the neural reflex arc of the Lacrimal Functional Unit (LFU) (Stern et al., 2004). This homeostatic mechanism ensures that a stable tear film moistens the ocular surface at all times. The lacrimal and meibomian glands, the conjunctival epithelium and goblet cells all receive a parasympathetic efferent nerve supply, but the role of the LFU in regulating the secretions of the non-lacrimal appendages is uncertain. For practical purposes, this chapter will refer to reflex tear secretion as if this were reflex lacrimal secretion.

There are other inputs to the lacrimal gland that act via the SSN in conjunction with those from the cornea, i.e. from the nasal mucosa, skin (not only pain but also including lid margin and ciliary/lash 'tickle'), retina (bright lights), acute pain and emotional stimuli, that not only influence lacrimal secretion but also determine the responsiveness of the gland to other sensory stimuli (Figure 6.1).

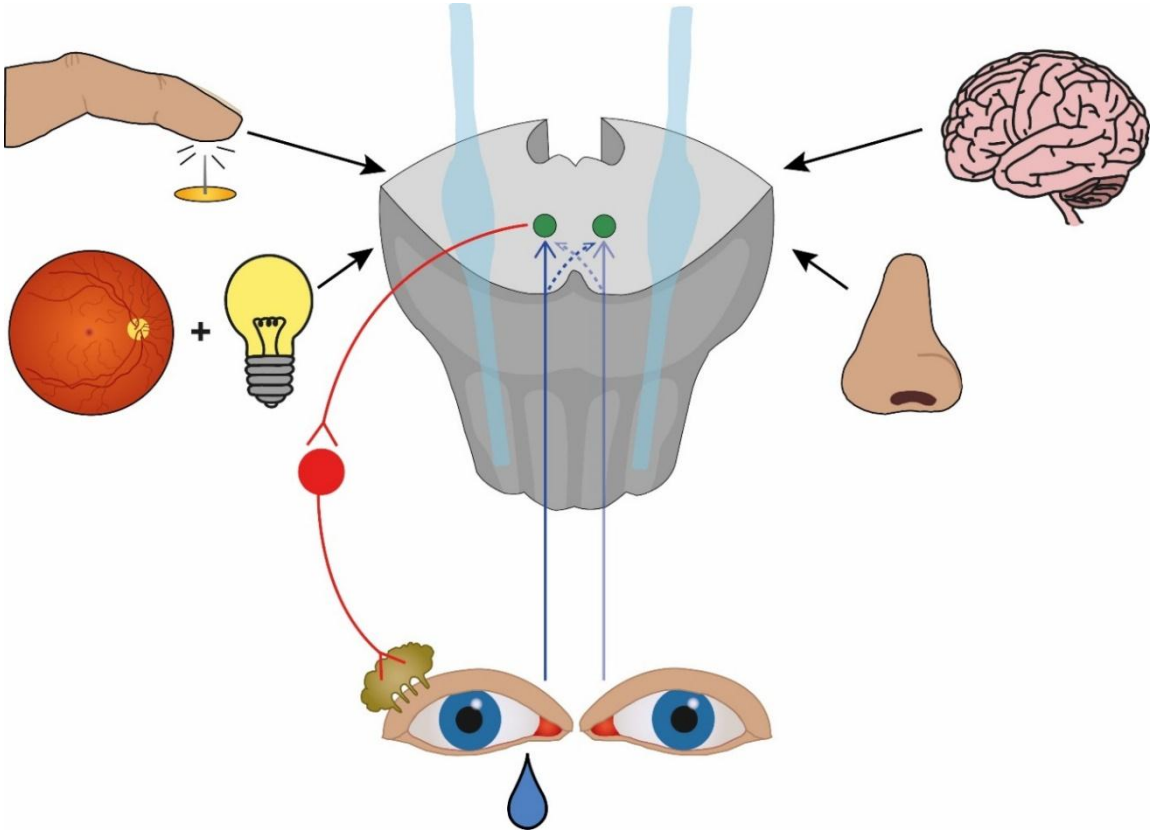


Figure 6.1 A schematic of the neural reflex arc and possible cross-sensory innervation input from the ipsilateral and contralateral afferents resulting in lacrimal gland stimulation. Additional inputs to lacrimal secretion include (clockwise from top left) skin, emotion, nasal mucosa, and retina.

Spontaneous blinking and the blink response are served by a similar reflex arc whereby the central connections of trigeminal neurones of corneal origin, synapse centrally in the nuclei of the IIIrd and VIIth cranial nerves, which give rise to somatic efferents whose activity determines the upstroke and the downstroke of the blink, respectively.

Bilateral topical anaesthesia reduces but does not abolish both lacrimal secretion and blinking, implying that there is an additional reflex contribution to these mechanisms (Lamberts, Foster and Perry, 1979; Jordan and Baum 1980; Pellegrini and Evinger, 1995; Li et al. 2012; Meng and Kurose, 2013). Emotional tearing is similarly dependent on the activity of higher centres. Importantly, the trigeminal neurones that supply the cornea also serve a trophic function which is essential to the turnover, maintenance and repair of the corneal epithelium, mediated for instance by the release of peptides such as nerve growth factor (NGF).

Loss of sensory drive to the lacrimal gland due to any form of trigeminal anaesthesia, including NK, results in a reduction of lacrimal secretion, which favours the occurrence of an aqueous-deficient dry eye (ADDE). A fall in blink rate, by extending the blink interval and increasing evaporative water loss, amplifies the degree of dry eye, and loss of sensation over the ocular surface may remove the protective action of the blink reflex.

Additionally, removing the trophic support of sensory neurones impairs the maintenance of a healthy ocular surface epithelium and its repair, and renders it vulnerable to external trauma. In NK, severity ranges from a punctate epithelial keratitis, to a persistent epithelial defect (PED) with severe inflammation, leading to an intractable corneal ulcer which may perforate. Because the eye is insensitive, minimising symptoms, diagnosis may be delayed.

When trigeminal anaesthesia is bilateral, it is predicted that the risk of precipitating an ADDE is high, since there is no opportunity for either lacrimal compensation or compensation of the blink response: lacrimal secretion and blink rate will both be reduced. When the trigeminal anaesthesia is unilateral, the extent to which a normal sensory function in the fellow eye compensates for and limits the risk of dry eye in the anaesthetic eye is not established, but ipsilateral aqueous deficient dry eye has been reported in this situation (Sacchetti and Lambiase, 2014). The hypothesis put forward here is that its occurrence will depend on the degree to which the central connections of afferents from one cornea influence efferent outputs from the contralateral lacrimal gland. This is referred to here as 'cross-innervation'. With a 50:50 level of cross-innervation, 50% of central afferents would feed into and synapse with ipsilateral, second order efferent neurones in the SSN and 50% would feed to the contralateral SSN. If cross-innervation is substantial then it would be expected that, with unilateral corneal anaesthesia, in conditions of ocular surface stress, such as exposure to desiccating environmental conditions or exposure to dust or chemical fumes, the reflex response from the normal, non-anaesthetic eye, would lead to a compensatory, reflex lacrimal response from both eyes. This would be protective to the anaesthetised eye. If there were no cross-innervation, then compensation would be confined to the normal eye and the anaesthetised eye would remain unprotected. In the absence of any reflex input to the lacrimal gland on the affected side, desiccation would be expected to drive tear osmolarity (tOsm) upwards, favouring the development of dry eye.

Along with acquired anaesthesia affecting reflex tearing, bilateral topical anaesthesia has also been shown to reduce but not abolish lacrimal secretion in both eyes, measured by the Schirmer I test (Lamberts, Foster and Perry, 1979; Jordan and Baum 1980; Li et al., 2012) or by fluorophotometry (Jordan and Baum, 1980). Since the nature of any sensory input from a normal cornea to the contralateral SSN is not known and its potential to influence contralateral lacrimal gland secretion is uncertain, the following experiments were designed to study the relative contribution of each eye to the reflex tear response, measured by the modified, sheathed, Schirmer I test (see Chapter 4) in controlled environmental conditions, after unilateral and bilateral topical anaesthesia.

6.2 HYPOTHESIS

It was proposed that in the steady state, when the eyes are open and exposed to the environment, sensory inputs from each eye stimulate lacrimal secretion both ipsilaterally and contralaterally via central connections of the trigeminal nerve with the SSN on each side. The study explored the effect of withdrawing sensory input, either by unilateral or bilateral topical anaesthesia, on the secretory responses of the two eyes, to learn how the output from the affected eye and that of the control fellow eye are modified.

6.3 MATERIALS AND METHODS

6.3.1 Subject enrolment

Eight subjects with normal eyes were recruited, 3 male and 5 female, aged 23.1 years ± 4.3 (mean ± SD) from students and staff at Anglia Ruskin University. Participants were individuals with a normal ocular surface by history and examination, according to defined criteria (see Chapter 2 for inclusion and exclusion criteria). After a preliminary visit, subjects were screened for the following inclusion criteria: tear osmolarity <308 mOsm/L (Sullivan et al., 2010); OSDI score <20; TBUT >10 seconds, and corneal staining <grade 2 using the Oxford clinical grading scale (Bron, Evans and Smith, 2003). Subjects were excluded if they had any active ocular disease, any clinically significant lid or conjunctival abnormalities (corneal scars or opacities), any clinically significant limbal or bulbar injection or conjunctival staining, any ocular surgery or injury within the previous 6 months, any systemic disease affecting ocular health, any nasolacrimal occlusion, or were using any topical ocular medications. Subjects were instructed not to use ocular cosmetics on the day of assessment and, if they were contact lens wearers, not to use their lenses for at least 8 hours before the procedure. Subjects six and seven also took part in the experiments featured in chapters four, five and six, and subjects four and five also took part in the experiments featured in chapter four. At least one month was allowed to elapse before these subjects were invited to participate in the other experiments. Table 6.1 contains the ocular profiles of the right eye of normal subjects recruited for this experiment.

Subject	Age (years)	Gender	tOsm (mOsm/L)	Corneal stain	Schirmer wetting (mm)	OSDI	TBUT (seconds)	Meibography
1	19	F	300	0	30	13.64	12.42	0
2	23	F	293	0	23	11.37	11.34	0
3	22	F	301	0	18	2.77	14.79	0
4	24	F	297	0	23	2.77	22.25	0
5	21	M	292	0	25	0	17.01	0
6	20	M	299	0	20	9.09	25.31	0
7	23	F	293	0	28	2.08	10.6	0
8	33	M	297	0	17	0	10.02	0
Mean ± SD								
	23.1 ± 4.32	5F:3M	296.5 ± 3.46	0 ± 0	23.0 ± 4.59	5.22 ± 5.35	15.49 ± 5.65	0 ± 0

Table 6.1 Profiles of the normal subjects consented for this study

6.3.2 Equipment

Studies were performed in a PSR "B" Series, Weiss Gallenkamp, controlled environment chamber (CEC), set to 'standard room conditions' based on guidelines (Workplace Health Committee Report 1998; Fang et al., 2004) at a temperature of 23°C, 45% relative humidity and 0.08 m/s airflow. As in the other studies, a period of 10 minutes was required for CEC conditions to be restored to their set values after the subject and examiner entered. Therefore, a period of 10 minutes after entry was allowed for CEC equilibration before the start of data collection.

The Schirmer I test without anaesthesia is considered to measure the reflex response of the lacrimal gland to ocular surface stimulation, acting through the LFU (Cho and Yap, 1993). This, therefore, is a measure of lacrimal secretory potential, assuming that the sensory and motor elements of the reflex apparatus are intact; this is referred to here as the reflex Schirmer test. The Schirmer I test can also be performed after instilling a topical anaesthetic, in which case, when it is performed bilaterally, in resting environmental conditions, it reflects the constitutive secretory activity of the lacrimal glands, modified by non-ocular inputs, sometimes referred to as basal tear secretion (Jones, 1966). It is referred to here as the Schirmer test with anaesthesia. In the current study, each subject underwent the Schirmer I test after topical anaesthesia bilaterally, and the Schirmer I test bilaterally, without anaesthesia, but proceeded by instillation of two saline drops as a control for the instillation of anaesthetic drops in the previous study. A further Schirmer I test was performed, with one eye receiving topical anaesthetic and the other, saline.

Because the instillation of a topical anaesthetic induces reflex tearing and the instilled volume itself could contribute to wetting of the Schirmer strip (Clinch et al., 1983; Afonso et al., 1999), the test is usually performed after removal of excess fluid from the conjunctival sac with an absorbent tissue. In this study, however, whether the Schirmer I test was conducted with anaesthesia or with saline, removal of excess fluid from the conjunctival sac (as opposed to the eyelid skin) was not performed because of the uncertainty of the adequacy of removal, and to avoid the effects of lid manipulations on tear production (Yokoi and Komuro, 2004). Instead, 10 minutes were allowed to elapse between drop instillation and performance of the test, on the basis that, in the presence of spontaneous blinking, excess fluid would be drained away over this period. This strategy is supported by the study of Bandlitz et al., (2014) which showed that meniscus curvature, a measure of tear volume (Yokoi et al., 2004), was restored to normal within 5 minutes of instillation of a 35µL drop of an artificial tear solution containing hydroxypropyl-guar and glycol (Bandlitz et al., 2014). The protocol used here involved instilling one drop of either a topical anaesthetic or of saline (Minims®) followed by another drop 30 seconds later. The average volume of each eye drop instilled was as follows: saline 41µL, tetracaine 34µL, and proxymetacaine 41µL. Using fluorophotometry, Mishima et al., (1966) determined that the average tear volume present in the eye was 6.2 ± 2.0 µL, and that the maximum capacity of the cul-de-sac for added volume was 30 µL; any fluid above this level was observed to overflow (Mishima et al., 1966). In this study, any overflow of fluid was dabbed away by the examiner, using a tissue applied to the lid margins and canthi while the eyes were closed. After the instillation of eye drops and allowing 10 minutes for drainage, the closed-eye (Serin et al., 2007), modified sheathed Schirmer test was then performed in both eyes of each subject (see Chapter 4). A Schirmer strip was hooked over the lid margin at its lateral third in the right eye, then the left, and the wetting length was recorded 5 minutes after the moment of insertion for each eye i.e. the left eye was read at a defined interval after the right eye. If full wetting occurred within the 5 minutes, the time at which this occurred was noted.

For topical anaesthesia, a procedure was adopted to achieve the minimum reflex tearing and the densest level of anaesthesia with the anaesthetics used (See Chapter 2 and 3 for details). A single drop of the short-acting anaesthetic 0.5% proxymetacaine (Minims®) was first instilled in the right eye and then in the left. This drop stings only slightly. This was followed, 30 seconds later, by 1 drop of 1% tetracaine (Minims®). To maximize spreading and mixing of the drops, the subject was asked to look up while each drop was instilled into the lower fornix and then asked to blink with the lower lid still drawn downward; this distributes the drop without overflow (Fraunfelder, 1976). Finally, the eyes were closed to allow excess fluid to be dabbed away from the lid margins and canthi (but not from the conjunctival sac). This sequence was followed by a period of 10 minutes of spontaneous blinking before any experimental procedure. This is in keeping with the

approach of Xu and Tsubota, who performed the anaesthetic Schirmer test at an interval of 5 minutes after drop instillation and without previous removal of tear fluid from the conjunctival sac (Xu and Tsubota, 1995). The same protocol was adopted for the instillation of saline, and the protocol followed for each phase of the experiment.

6.3.3 Protocol

Subjects made two visits on separate days; Figure 6.2 illustrates the timeline of each visit and Table 6.2 the order of the experiments for each subject. The first visit lasted approximately 1 hour. The subject and the examiner entered the CEC, and after seating the subject comfortably, saline was instilled into each eye as per protocol, and was followed, after an interval of 10 minutes, by a Schirmer test. After this, the examiner and the subject remained in the CEC for a 10-minute recovery period. This recovery period was over three times longer than that considered sufficient for the eye to recover after multiple Schirmer tests and obtain independent measures, as specified in the Loran et al., (1987) study. It was then randomly decided whether the subject would undergo either unilateral or bilateral afferent blockade and the protocol for instillation of the appropriate eye drops was carried out. The second visit occurred on a subsequent day and lasted approximately 30 minutes. After entering the CEC, the subject received either unilateral or bilateral anaesthesia depending on which regime had been undertaken at the previous visit. Subjects were masked as to whether they were receiving topical saline or anaesthetic, or the combination, but the same examiner was responsible for drop instillation and Schirmer test measurements. A slit-lamp examination was performed following all procedures to check for adverse events. An example record card for this experiment is shown in Appendix X.

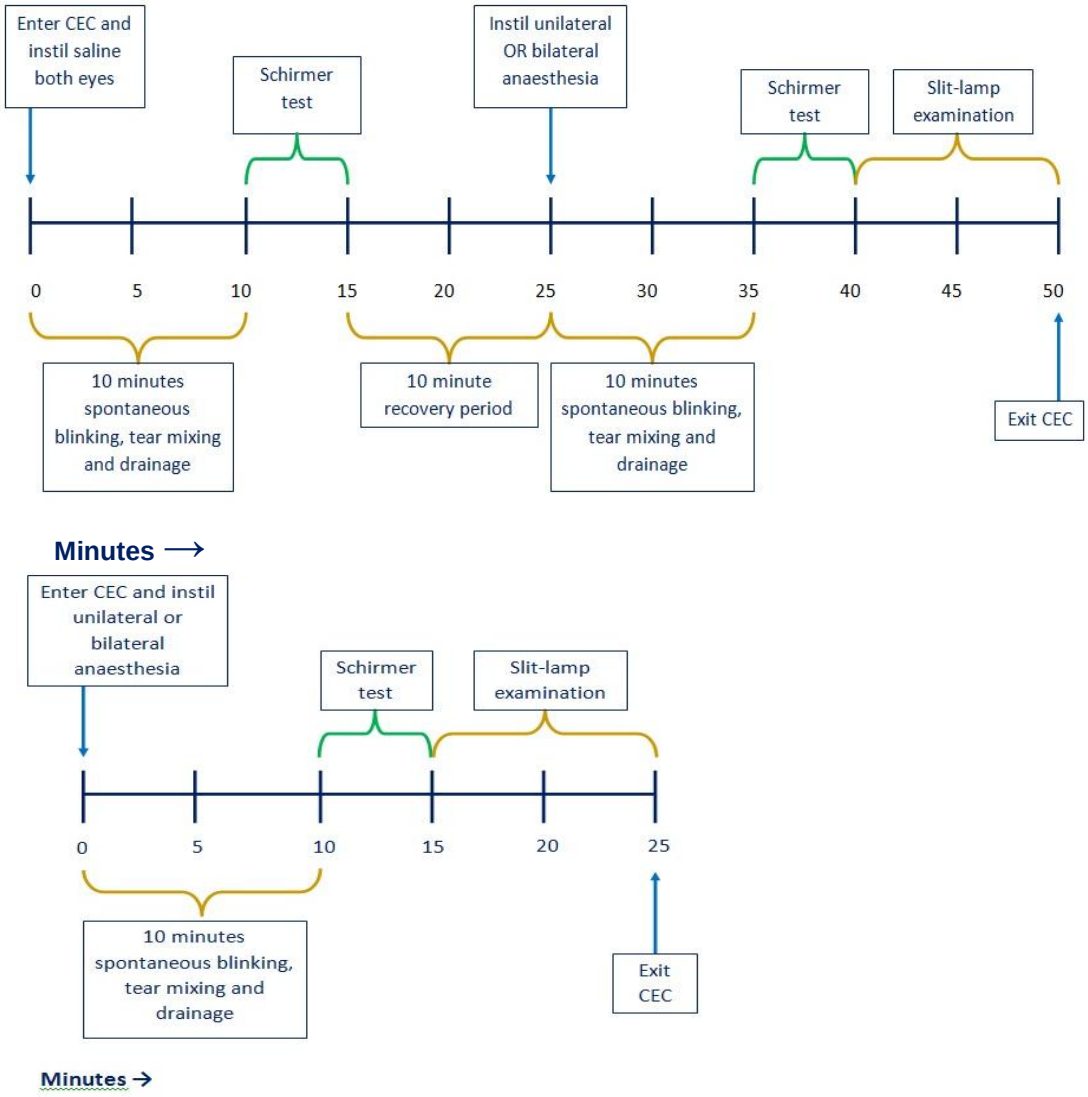


Figure 6.2 Timeline of bilateral saline, bilateral anaesthetics or unilateral saline and anaesthetic visits.

Subject	Age	Gender	Visit 1	Visit 2	Experimental Key
1	19	F	¥ §	¶	¥ = *Saline control phase:* At zero time, one drop of saline was instilled into each eye, followed by another at 30 seconds to control for subsequent anaesthetic studies. § = *Unilateral afferent block phase:* one drop of proxymetacaine 0.5% was instilled into one eye (randomly selected) and a drop of saline into the fellow eye, followed after 30 seconds by tetracaine 1% into the first eye and saline into the fellow eye. ¶ = *Bilateral afferent block phase:* one drop of proxymetacaine 0.5% is instilled into both eyes, followed after 30 seconds by tetracaine 1% into both eyes.
2	23	F	¥ ¶	§	
3	22	F	¥ §	¶	
4	24	F	¥ ¶	§	
5	21	M	¥ ¶	§	
6	20	M	¥ ¶	§	
7	23	F	¥ §	¶	
8	33	M	¥ ¶	§	

Table 6.2 Summary of experimental design for each subject.

6.3.4 Statistical Analysis

The differences in Schirmer wetting lengths when compared across the three conditions were analysed using a repeated measures one way ANOVA with simple contrasts. The Bonferroni method was used to make adjustments for multiple comparisons. The differences in Schirmer wetting lengths within the individual conditions were analysed using a paired t-test. The Shapiro-Wilk test was used to evaluate normality of distribution. Differences were considered statistically significant with *P* values less than 0.05. Data is presented as mean ± standard deviation. Analyses were performed using SPSS 20.

6.4 RESULTS

Eight subjects with normal eyes were recruited; three male and five female, aged 23.1 years ± 4.3 (mean ± SD). The Schirmer wetting test scores for all eight participants in each phase of the experiment are presented in Table 6.3 and Figure 6.3. To make data more accessible, the eye receiving anaesthesia in the unilateral experiment was designated 'Eye 1' and the fellow eye was designated 'Eye 2', throughout the study.

The Schirmer wetting lengths in the saline control experiment were: Eye 1 = 23.1 ± 7.2mm; Eye 2 = 24.9 ± 4.1mm. There was no significant difference in between-eye wetting lengths: 1.8 ± 5.4mm (p = 0.394). In the unilateral anaesthesia experiment, the average wetting length was 11.3mm ± 4.1 in the anaesthetised eye, and 19.4mm ± 7.4 in the saline control eye. The wetting length was reduced in the anaesthetised eye when compared to the control (bilateral saline experiment) by 11.9 ± 4.0mm (p = <0.0005), and its fellow eye by 8.1 ± 5.6mm (p = 0.005). The fellow eye response was also reduced compared to its control (bilateral saline experiment) by 5.5 ± 5.7mm, however this did not quite reach significance (p = 0.06). In the bilaterally anaesthetised eyes the average wetting length was 8.7 ± 5.2mm in Eye 1 and 10.0 ± 3.8mm in Eye 2. There was no significant difference in the between-eye wetting lengths: 1.3 ± 2.4mm (p = 0.171). The wetting length was reduced in both eyes after bilateral anaesthesia compared to their bilateral saline controls (p = 0.0003; p = <0.0005).

Subject	Saline control n = 8		Unilateral anaesthesia n = 8		Bilateral anaesthesia n = 8	
	Schirmer wetting length – mm					
	Eye1	Eye2	Eye1	Eye2	Eye1	Eye2
1	**33**	30	**18 RE**	25	**7**	10
2	**24**	28	**12 LE**	15	**4**	7
3	**16**	21	**5 LE**	15	**4**	5
4	**15**	23	**8 LE**	12	**5**	7
5	**17**	25	**10 RE**	23	**7**	9
6	**20**	18	**9 RE**	15	**10**	12
7	**28**	29	**15 RE**	34	**14**	16
8	**32**	25	**13 RE**	16	**18**	14
Mean wetting length	23.1 ± 7.2	24.9 ± 4.1	11.3 ± 4.1	19.4 ± 7.4	8.7 ± 5.2	10.0 ± 3.8
Mean difference in Schirmer wetting length & significance	1.8 ± 5.4mm *p* = 0.394		8.1 ± 5.6mm *p* = 0.005		1.3 ± 2.4mm *p* = 0.171	
Percentage change in Schirmer wetting length	Eye 1 unanaesthetised 23.1 ± 7.2 vs. Eye 1 anaesthetised 8.7 ± 5.2		62.4%		**Average 61.1%**	
	Eye 2 unanaesthetised 24.9 ± 4.1 vs. Eye 2 anaesthetised 10.0 ± 3.8		59.8%			

Table 6.3 Summary of raw data for all subjects.

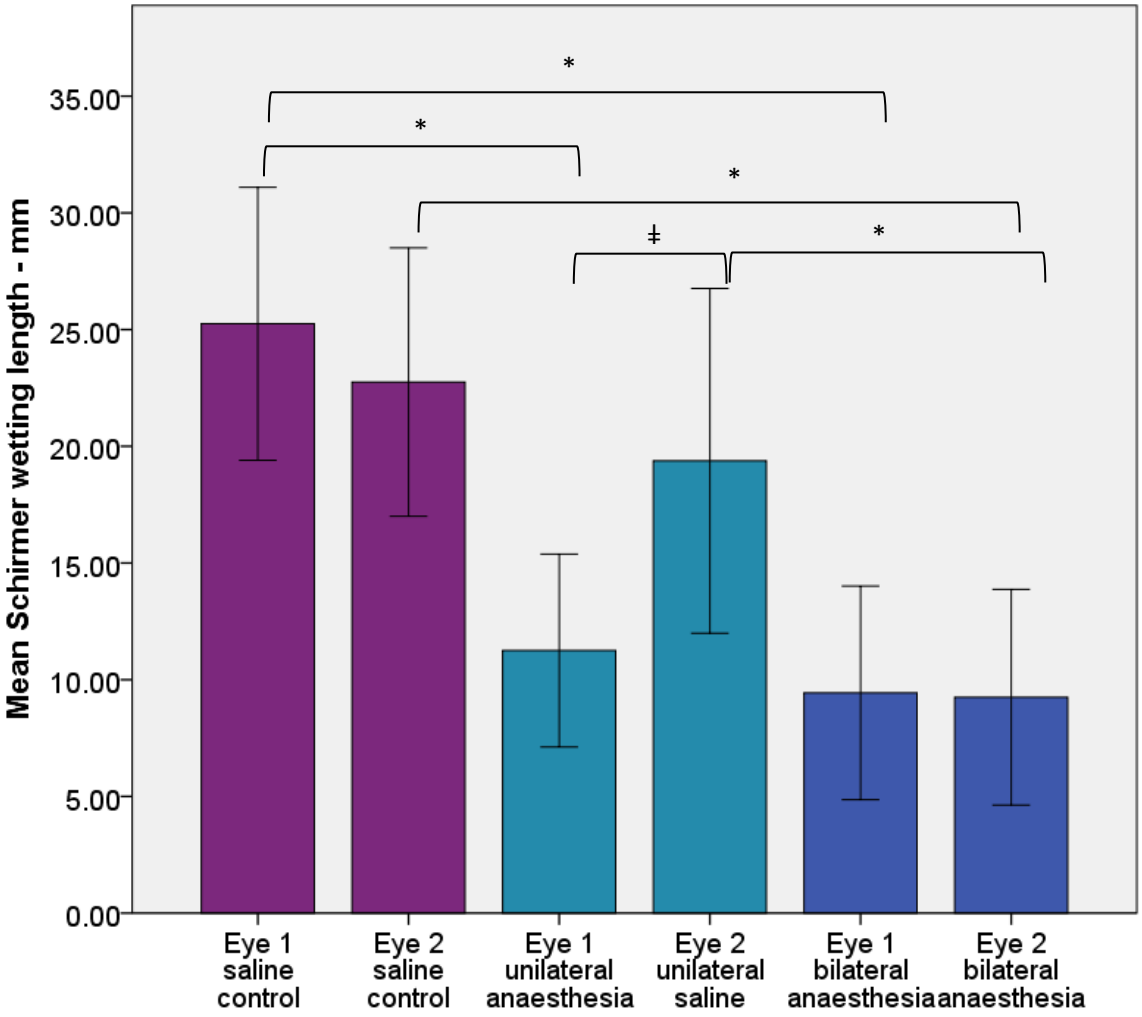


Figure 6.3 Schirmer wetting test scores for all 8 participants, after bilateral saline, unilateral anaesthesia, and bilateral anaesthesia phases. ǂ Statistically significant difference from the fellow eye (paired t-test).
* Statistically significant difference between eye 1 and eye 2 between each experiment (repeat measures one way ANOVA). Values are mean ± SD.

6.5 DISCUSSION

The focus of this study was to determine whether there is sensory cross-connectivity between the cornea of one side and the opposite superior salivatory nucleus (SSN). It has yet to be investigated in humans as to whether the concept of cross-connectivity exists and this experiment was designed to elucidate this topic. It is a familiar experience that a foreign body in one eye can induce tearing from both eyes. Is this due to sensory cross-connectivity of the kind discussed here, or is it mediated through pain pathways? The same can be asked concerning irritation of the nasal mucosa, where, when performing the nasolacrimal Schirmer II test, the response of both eyes is recorded after stimulation of the nasal mucosa on one side only (Tsubota, 1998). In the current study it was confirmed, as in other reports that bilateral topical anaesthesia results in a bilateral reduction of the Schirmer wetting length compared with the reflex Schirmer response in

control eyes (Siganos et al., 2002; Lamberts, Foster and Perry, 1979; Jordan and Baum, 1980; Konomi et al., 2008; Jones, 1966). The wetting length of the strip was also shorter after bilateral anaesthesia than after unilateral anaesthesia, although there was no significant difference between these values (11.3mm-unilateral and 8.7mm-bilateral p = 0.60). This is in line with a previous study by Jordan and Baum showing evidence that when sensory input is decreased by the use of topical anaesthesia, tear secretion is reduced (Jordan and Baum, 1980). It was found that unilateral topical anaesthesia resulted in an ipsilateral reduction of Schirmer wetting of a similar degree. It can be interpreted that these results were due to the withdrawal of the reflex sensory drive from the ocular surface to the ipsilateral SSN and lacrimal gland. However, some interpretative caution must be exercised because instilled anaesthetic drops, which drain into the nasolacrimal duct, are likely to cause some degree of anaesthesia of the nasal mucosa, and the study of Gupta et al., indicated that nasal anaesthesia alone can inhibit lacrimal secretion (Gupta, Heigle and Pflugfelder, 1997). Therefore, the possibility arises that some of the effect of topical ocular anaesthesia on lacrimal secretion could be due to loss of sensory drive from the nose. Heigle and Pflugfelder (1996) demonstrated an ipsilateral reduction in the reflex Schirmer response of 90.8%, compared with normal controls, in patients with unilateral neurotrophic keratitis (NK), in whom nasal mucosal anaesthesia was combined with dense corneal anaesthesia (Heigle and Pflugfelder, 1996). This contrasted with the findings in four patients with *herpes zoster ophthalmicus* (HZO) without keratitis, in whom, although there was a moderate reduction in corneal sensitivity, nasal sensation was normal and there was no reduction in the reflex Schirmer response with or without nasolacrimal stimulation. They concluded that there was an ipsilateral, sensory stimulus to lacrimal secretion arising from the nasal mucosa. These authors also showed that the Schirmer wetting length was reduced by 60.7% in the contralateral eyes of those patients with NK, which they considered to be evidence of sensory cross-innervation from the nasal mucosa of one side, to the SSN of the opposite side. It could not be excluded, however, that the original or activated infection had caused subclinical nerve damage on the clinically unaffected side.

This was supported by a further study by this group, in which the Schirmer wetting length, compared with baseline, was reduced to a similar degree on both sides, after unilateral nasal anaesthesia induced by insufflation (36.4% on the ipsilateral and 29.7% on the contralateral side) (Gupta, Heigle and Pflugfelder, 1997). These authors concluded that sensory stimulation of the nasal mucosa was an important contributor to basal tear production. Because the effects were demonstrated using the reflex Schirmer I test, which is not relevant to basal tear secretion, it would be more appropriate to conclude that this study provided evidence for modulatory influence of nasal sensory input on the reflex tear response, with evidence of cross-innervation. The studies by Heigle and Pflugfelder

(1996), Gupta et al., (1997) (Heigle and Pflugfelder, 1996; Gupta, Heigle and Pflugfelder, 1997), and the current study, used the Schirmer test to measure reflex tear flow in the clinic, for reasons of cost and technical difficulty. This is a test both of the integrity of the reflex arc of the lacrimal functional unit (LFU), and of the functional state of the lacrimal gland, without the possibility of determining their relative contributions to the outcome. In the normal eye, the Schirmer paper tip stimulates the ocular surface, increases afferent drive and results in a reflex lacrimal tear response. The volume of secretion will therefore be higher than the resting tear secretion. Thus the Schirmer test can only be used in a limited way to explore this situation because the reflex Schirmer test is a measure of the response of the eye to a particular irritative stimulus to the ocular surface. The test does not provide any information about the tear secretory status of the eye in everyday environmental conditions but merely the ability of the lacrimal apparatus to respond to a mechanical and nociceptive stimulus of that magnitude. Such information can only be obtained by techniques such as fluorophotometry, which allow tear secretion to be measured in a controlled environment.

The existence of sensory cross-innervation is relevant to the health of the fellow eye in conditions that affect the sensory innervation of the ocular surface unilaterally, such as unilateral refractive surgery, *Herpes simplex* keratitis (HSK), HZO, and in NK from whatever cause. The aim of this study was to explore the contribution of each eye to the Schirmer response of its fellow eye, measured after unilateral and bilateral topical anaesthesia. It was hypothesised that in the steady state, when the eyes are open and exposed to the environment, sensory inputs from each eye stimulate lacrimal secretion both ipsilaterally and contralaterally through central connections of the trigeminal nerve with the SSN on each side. This sensory drive would be concerned with maintaining a steady state in a given environment, and by withdrawing one of the major sensory inputs through corneal topical anaesthesia, it was hoped to understand how the output would be modified. There is evidence for contralateral, central trigeminal projections in animals, but this cannot be taken as supportive of such a condition in humans (van der Werf et al., 1996; Clarke and Bowsher, 1962; Pfaller and Arvidsson, 1988; Jacquin, Chiaia and Rhoadest, 1990). If there were a significant level of central, sensory cross-innervation, then it could be predicted that when the Schirmer test is performed simultaneously in the two eyes, with one eye anaesthetised and the other not, the fall in reflex secretion by the anaesthetised eye would be offset by an afferent drive from the unanaesthetised fellow eye; and conversely, in the unanaesthetised eye, the reflex response would be reduced, owing to a lack of drive from the now anaesthetised fellow eye. The results here demonstrated a reduction in the Schirmer wetting length after bilateral topical anaesthesia, averaged for the two eyes, of 61.1% (Table 6.3). This compares to a reduction of the Schirmer test response of between 28% and 53% reported in the

literature (Siganos et al., 2002; Lamberts, Foster and Perry, 1979; Jordan and Baum, 1980; Li et al., 2012). After unilateral topical anaesthesia in this study, the effects on the Schirmer wetting length were in the direction predicted by an assumption that there is sensory cross innervation to the SSN, that is the fall in secretion in the anaesthetised eye was less than that which occurred when there was bilateral anaesthesia, and the wetting length in the fellow unanaesthetised eye was lower than in its control eye after the bilateral Schirmer test, after saline. However, the differences shown in each case were not significant ($p = 0.6$; $p = 0.06$). Because the numbers were small, this does not entirely exclude the existence of sensory cross-innervation. However, the possibility that those topical anaesthetics may induce a degree of nasal mucosal anaesthesia, which could itself reduce the secretory drive to the lacrimal gland of a fellow eye, could weigh further against the existence of cross-innervation from the cornea to the contralateral SSN.

A number of studies in the literature could be interpreted as supportive of ocular cross-innervation. Several investigators have reported a bilateral reduction in the reflex Schirmer response in the presence of unilateral HSK, noting that the diminished response in the fellow eye was often not significantly different from that in the affected eye (Simard-Lebrun et al., 2010; M'Garrech et al., 2013; Keijser et al., 2002). However there is evidence that in both HSK and HZO, unilateral clinical disease is accompanied by contralateral loss of trigeminal innervation of the clinically unaffected cornea. The Hamrah group has compared clinical indices in the affected and unaffected eyes of 25 patients with unilateral HSK (Hamrah et al., 2010) and 27 patients with unilateral HZO with those in healthy controls (Hamrah et al., 2013). In each condition, they demonstrated a significant decrease in a range of sub-basal nerve parameters, not only in the affected eye but also, to a lesser degree, in the clinically unaffected eye. These observations are consistent with the findings in patients with herpes zoster infections at other sites in the body (Clarke and Bowsher, 1962; Watson et al., 1991). Regardless of the basis for the involvement of contralateral sensory neurones after clinically unilateral herpes simplex or herpes zoster infections, the finding of a reduced contralateral Schirmer response in such conditions cannot be interpreted as evidence for sensory cross-innervation in the ophthalmic division of the trigeminal nerve.

However the influence of trigeminal sensory deprivation, confined to a single cornea, on the reflex Schirmer response, can be examined in another clinical scenario. Various forms of refractive surgery (e.g. photorefractive keratectomy and LASIK) cause loss of corneal sensitivity for a period of 6 to 12 months after the procedure (Özdamar et al., 1999; Aras et al., 2000; Siganos et al., 2002; Battat et al., 2001), and a small number of studies have demonstrated a reduction in the reflex Schirmer response after such procedures. In healthy individuals, in whom the lacrimal glands and the neural pathways of the LFU may be assumed to be intact, any effect of such surgery on the ipsilateral

reflex Schirmer response may be interpreted as a result of a blockade of the afferent limb of the LFU on the side of surgery. (See Table 6.4 for a summary of the results for the above studies.) These various reports support the view that, in human subjects, corneal anaesthesia alone leads to an ipsilateral reduction of lacrimal secretion by decreasing the sensory drive from the cornea to the ipsilateral lacrimal gland, independent of any action on the nasal mucosa. There is also a suggestion of a graded response to corneal anaesthesia, because in one study comparing refractive lenticule extraction (using the femtosecond laser) with LASIK, the effect of surgery on corneal sensitivity was smaller in the former procedure, and the reduction of the reflex Schirmer response was less (Gao et al., 2014). Similarly, in studies of LASIK surgery, the Schirmer wetting length was further reduced after an anaesthetic Schirmer test, which suggests that refractive surgery does not cause complete corneal anaesthesia and the effect can be fortified by the addition of topical anaesthesia (Siganos et al., 2002; Konomi et al., 2008). In the current study, however, as noted, we cannot exclude an effect due to a degree of coincident, nasal anaesthesia. Overall, the proposition of Heigle and Pflugfelder that; 'Perhaps lacrimal gland stimulation results from the sum of sensory inputs from the adnexal skin, cornea, nasal mucosa, contralateral eye and even central stimulation' may be accepted (Heigle and Pflugfelder, 1996), although the current study indicates that if there is an input from the contralateral ocular surface, it is limited compared with that from the contralateral nasal mucosa. This result is likely to be due to the small number of subjects recruited into the study. The effects of inputs that maintain lacrimal secretion in non-stressful environmental conditions should be distinguished from those that occur in more extreme environmental conditions such as exposure to noxious chemical and physical stimuli, when it may be supposed that additional pathways are recruited.

It is still of great importance to discover more about the afferent sensory connections to the SSN from one eye to the efferent lacrimal output of the fellow eye, as there are many clinical situations that result in unilateral anaesthesia, and further research is planned with expanded subject numbers, to clarify the concept of cross-connectivity.

Measure	Subjects	n	Findings	% change	Authors
Schirmer test: mm in 5 minutes					
Unanaesthetised and anaesthetised Schirmer	• Normal patients	n=223 (N)	Unanaesthetised Schirmer 19.6±9.2 vs. Anaesthetised Schirmer 11.9±7.8	39.3%	Lamberts et al., 1979
Unanaesthetised and anaesthetised Schirmer	• Young patients (25-45years) • Old patients (57-71 years)	n=15 (N)	Young, unanaesthetised Schirmer 30±6 vs. Young, anaesthetised Schirmer 14±6 Old, unanaesthetised Schirmer 17±12 vs. Old, anaesthetised Schirmer 9±6	53% 47%	Jordan and Baum, 1980
Unanaesthetised Schirmer	• Affected and unaffected eyes of NK patients • HZO patients • Normal controls	n = 19 (NK=5, HZO=4, N=10)	NK 2.60±3.29 vs. Fellow 11.2±6.57 NK 2.60±3.29 vs. HZO 30.75±7.22 NK 2.60±3.29 vs. Normal 28.5±8.03 Fellow 11.2±6.57 vs. Normal 28.5±8.03	76.8% 91.5% 90.8% 60.7%	Heigle and Pflugfelder, 1996
Unanaesthetised Schirmer following anaesthesia of R nasal mucosa	• Normal patients	n=12 (N)	Baseline 23.71mm vs. Ipsilateral eye 15.09mm Baseline 22.69mm vs. Contralateral eye 15.95mm Saline 22.91mm vs. Ipsilateral eye 15.09mm Saline 19.36mm vs. Contralateral eye 15.95mm	36.4% 29.7% 34.1% 17.6%	Gupta et al., 1997
Unanaesthetised Schirmer	• PRK eye • Unoperated fellow eye	n=32 (PRK)	PRK eye 14.45±7.79 vs. Unoperated eye 28.33±5.89	49%	Ozdamar et al., 1999)
Unanaesthetised Schirmer	• LASIK eye post-op* • Unoperated fellow eye	n=28 (LASIK)	LASIK eye 16.17±2.50 vs. Unoperated eye 21.07± 7.08	23.26 %	Aras et al., 2000
Unanaesthetised Schirmer	• LASIK patients pre-op and 1m post-op	n=48 (LASIK)	Pre-op 23.95±10.05 vs. 1m post-op 18.06±10.42	24.6%	Battat et al., 2001
Unanaesthetised Schirmer vs. Anaesthetised Schirmer	• LASIK patients pre-op and 1m, 3m and 6m post-op	n=42 (LASIK)	Pre-op 16.2±4.69 vs. 1m post-op 12.8±3.15 Pre-op 16.2±4.69 vs. 3m post-op 15.25±4.2 Pre-op 16.2±4.69 vs. 6m post-op 15.96±1.2 Pre-op unanaesthetised 16.2±4.69 vs. Pre-op anaesthetised 11.6±3.45	21% 5.9% 1.5% 28.4%	Siganos et al., 2002
Unanaesthetised and anaesthetised Schirmer	• LASIK patients pre-op	n=18 (pre-LASIK)	Unanaesthetised 24.53±3.34 vs. anaesthetised 15.57±1.76	36.5%	Konomi et al., 2008
Unanaesthetised Schirmer	• HSK affected eye • HSK unaffected fellow eye • Normal controls	n=24 (HSK)	HSK affected 12.2±9.9 vs. Control 19.1±10.2 HSK Unaffected eye 12.8±8.4 vs. Control 18.5±10.6	36.1% 30.8%	Simard-Lebrun et al., 2010
Unanaesthetised and anaesthetised Schirmer	• ADDE • EDE	Dry Eye n=110 (ADDE=68, EDE=42)	ADDE unanaesthetised 5mm˜ vs. ADDE anaesthetised 3mm˜ EDE unanaesthetised 14mm˜ vs. EDE anaesthetised 6mm˜	40%˜ 57%˜	Li et al., 2012
Unanaesthetised Schirmer	• HSK affected eye • HSK unaffected fellow eye • Normal controls	n=35 (HSK)	HSK affected 15.9±3.1 vs. Control 20.3±1.8 HSK Unaffected eye 17.3±2.9 vs. Control 20.8±1.7	21.7% 16.8%	M'Garrech et al., 2013
Unanaesthetised Schirmer	• LASIK patients pre-op and 1w, 1m and 3m post-op	n=32 (LASIK)	Pre-op 13mm˜ vs. 1w post-op 9mm˜ Pre-op 13mm˜ vs. 1m post-op 9˜ Pre-op 13mm˜ vs. 6m post-op 10˜	30.7% 30.7% 23.1%	Gao et al., 2014
Unanaesthetised and anaesthetised Schirmer	• Normal patients	n=8 (N)	Eye 1 unanaesthetised 23.13±7.22 vs. Eye 1 anaesthetised 8.69±5.20 Eye 2 unanaesthetised 24.88±4.12 vs. Eye 2 anaesthetised 10.00±3.78	62.4% 59.8%	Current study

Table 6.4 Summary of unilateral anaesthesia studies.

Measure	Subjects	n	Findings	% change	Authors
In Vivo Confocal Microscopy: number of nerve trunks					
Total number of nerve trunks and branches in a coronal section, (460 x 345µm)	• HSK affected eye • HSK unaffected fellow eye Normal controls	n=25 (HSK)	HSK affected 5.2±4.5 vs. Control 13.1±3.8 HSK Unaffected eye 7.8±3.3 vs. Control 13.1±3.8	60.3% 40.5%	Hamrah et al., 2010
Total number of nerve trunks and branches in a coronal section (460 x 345µm)	• HZO affected eye • HZO unaffected fellow eye Normal controls	n=27 (HZO)	HZO affected 5.4±2.8 vs. Control 13.1±3.8 HSK Unaffected fellow 8.3±2.9 vs. Control 13.1±3.8	58.8% 36.6%	Hamrah et al., 2013
Fluorophotometry: tear turnover %/minute					
Fluorophotometry with and without anaesthesia	• Young patients (25-45years) Old patients (57-71 years)	n=15 (N)	Young, unanaesthetised 0.9±0.4 vs. Young, anaesthetised 0.2±0.1 Old, unanaesthetised 0.9±0.2 vs. Old, anaesthetised 0.4±0.2	77% 55%	Jordan and Baum, 1980
Fluorophotometry without anaesthesia	• HSK affected eye • HSK unaffected fellow eye Normal controls	n=16 (HSK)	HSK affected 7.9±4.9 vs. Control 14.3±6.5 HSK unaffected fellow 7.9±5.6 vs. Control 14.3±6.5 HSK affected 7.9±4.9 vs. HSK unaffected eye 7.9±5.6	44.8% 44.8% 0%	Keijser et al., 2002
Central corneal sensitivity: mm					
Cochet-Bonnet	• LASIK patients pre-op and 1w, 1m and 3m post-op • ReLEx patients pre-op and 1w, 1m and 3m post-op	n=47 (LASIK=32, ReLEx=15)	Pre-op 58mm˜ vs. 1w post-op 20mm˜ Pre-op 58mm˜ vs. 1m post-op 22mm˜ Pre-op 58mm˜ vs. 3m post-op 30mm˜ Pre-op 58mm˜ vs. 1w post-op 50mm˜ Pre-op 58mm˜ vs. 1m post-op 52mm˜ Pre-op 58mm˜ vs. 3m post-op 56mm˜	65.5% 62.1% 48.3% 13.8% 10.3% 3.4%	Gao et al., 2014
Cochet-Bonnet	• LASIK patients pre-op and 1w, 1m and 6m post-op	n=48	Pre-op 36mm˜ vs. 1w post-op 3mm˜ Pre-op 36mm˜ vs. 1m post-op 18mm˜ Pre-op 36mm˜ vs. 6m post-op 12mm˜	91.7% 50.0% 66.7%	Battat et al., 2001
Central corneal sensitivity: gm/cm^2					
Cochet-Bonnet	• LASIK patients pre-op and 1w, 3m and 9m post-op	n=24	Pre-op 1gm/cm$^{2\sim}$ vs. 1w post-op 2.3gm/cm$^{2\sim}$ Pre-op 1gm/cm$^{2\sim}$ vs. 3m post-op 1.5gm/cm$^{2\sim}$ Pre-op 1gm/cm$^{2\sim}$ vs. 9m post-op 1 gm/cm$^{2\sim}$	56.5% 33.3% 0.0%	Konomi et al., 2008
ADDE=aqueous deficient dry eye; EDE=evaporative deficient dry eye; HSK=*Herpes simplex keratitis;* HZO=*Herpes zoster ophthalmicus*; LASIK=Laser-Assisted In-Situ Keratomileusis; N=normal; NK=neurotrophic keratitis; PRK=photorefractive keratectomy; ReLEx=Refractive Lenticule Extraction ˜Approximate from figures; * Timescale of test not stated					

Table 6.4 (Continued) Summary of unilateral anaesthesia studies.

The material presented here is reported in the publication: Willshire, C., Buckley, R.J. and Bron, A.J., 2017. Central Connections of the Lacrimal Functional Unit. *Cornea,* 36 (8), pp.898-907. This article is contained in Appendix XIV.

Chapter 7:

THE EFFECT OF DESICCATING STRESS ON TEAR OSMOLARITY WITH AND WITHOUT SENSORY BLOCKADE

The experiments described in this chapter were designed to study the effect of desiccating stress (low humidity) on tear osmolarity (tOsm) in open eye conditions, with and without sensory blockade, in normal and dry eye disease (DED) subjects.

7.1 INTRODUCTION

Osmolar homeostasis of the tears is closely regulated by the lacrimal functional unit (LFU) and is considered to be the primary drive for homeostasis of the tear film (Mathers, 2000). Tear production is up- or down-regulated in response to changes in sensory inputs from the ocular surface representing increased or decreased environmental stress. Desiccating stress can result in ocular surface damage in the form of staining (Abelson et al., 2012; Alex et al., 2013). There is an increased likelihood of this response in DED patients who exhibit greater than normal evaporation from the tear film, resulting in higher tOsm (Craig et al., 2000), or who, with their compensatory system already partially engaged by the disease, are unable to compensate further. Injury to the epithelial cells as a result of hyperosmolarity stimulates an increased sensory response from the corneal nerves via the LFU. As DED progresses, a state of corneal hypersensitivity is thought to exist that reduces the threshold for stimulation and results in a compensatory increase in lacrimation and blinking but this phase is followed by corneal hyposensitivity (Xu et al., 1996; De Paiva and Pflugfelder, 2004; Bourcier et al., 2005; Tuisku et al., 2008) and a consequent loss of compensation. This is considered to amplify the level of tOsm in the later stages of DED (Bron et al., 2009).

In recent studies in which normal and DED subjects were exposed to 1 or 2 hour periods of desiccating stress at 5% relative humidity (RH), an increase in corneal staining occurred but was not accompanied by a significant increase in tOsm (Abusharha and Pearce, 2013; Teson et al., 2013; López-Miguel et al., 2014). One explanation for this could be that although induced tear hyperosmolarity at the corneal surface was the basis of the corneal damage in the blink interval, compensatory reflex tearing had diluted tOsm overall, so that a rise did not occur in the meniscus. It is argued here, that if sensory drive from the ocular surface were to be blocked by topical anaesthesia, in conditions of desiccating stress, absence of the compensatory increase in tear production would allow

an increase in tOsm to occur that would be detected in the meniscus. This is the basis of the research presented below.

7.2 HYPOTHESIS

It was hypothesised that desiccating stress to the ocular surface, in the absence of a compensatory, reflex tear response via the LFU, would result in a rise in tOsm detectable in the tear meniscus. A secondary proposition was that this would be accompanied by signs of epithelial corneal damage in the form of a punctate epithelial keratopathy. In the experiments described below, desiccating stress was induced by exposure to a low RH and epithelial damage was quantified by observing punctate epithelial staining. Since the responses might be expected to differ in normal subjects and patients with DED, both groups of subjects were studied. Subjects were exposed to two sessions of desiccating stress in the form of an RH of 5%, for a period of 60 minutes each. In one session, both eyes were anaesthetised with topical anaesthetic and in the other, both eyes received a saline control drop. It was predicted that tOsm would rise significantly during the period of topical anaesthesia and afferent blockade, likely to be in the order of 30 minutes duration.

7.3 MATERIALS AND METHODS

7.3.1 Subject enrolment

Eight subjects with normal eyes were recruited, 4 male and 4 female, aged 37.0 years ± 23.57 (mean ± SD) from students and staff at Anglia Ruskin University. Participants were individuals with a normal ocular surface by history and examination, according to defined criteria (see Chapter 2 for inclusion/exclusion criteria). Subjects three and four also took part in the experiments featured in chapters four, five and six, and subjects six and seven also took part in the experiments featured in chapter four. At least one month was allowed to elapse before these subjects were invited to participate in the other experiments.

Eight patients with DED were recruited, 2 male and 6 female (4 diagnosed with Sjögren Syndrome), aged 58.88 years ± 17.13 (mean ± SD) from staff at Anglia Ruskin University and from the Cambridgeshire British Sjögrens Syndrome Association (BSSA) group. The Sjögren patients who volunteered for this study also participated in the cross-connectivity study reported in Chapter 5; there was at least two months between the data collection of the two experiments. DED patients were individuals who fulfilled the internationally accepted definition of DED (Lemp et al., DEWS I 2007) by history and by ocular surface examination (see Chapter 2 for screening inclusion/exclusion criteria). All but one of the DED patients were the same as those recruited for the experiment outlined in Chapter 4; a

profile of their DED status at recruitment can be viewed in this chapter. Table 7.1 contains the ocular profiles of the right eye of subjects recruited for this experiment.

Parameter Mean ± SD (min-max)	Subjects		
	Normal (n=8)	Sjögren syndrome DED (n=4)	Non Sjögren syndrome DED (n=4)
Gender	5 Male : 3 Female	4 Female	2 Male : 2 Female
Age (years)	40.00 ± 24.6	66.75 ± 7.93	50.75 ± 21.03
tOsm (mOsm/L)	294.0 ± 3.66	314.3 ± 11.21	313.75 ± 4.86
Corneal staining	1.25 ± 1.16	3.5 ± 2.38	2.0 ± 0.0
Schirmer wetting (cm)	23.3 ± 7.19	5.3 ± 2.5	7.0 ± 2.16
OSDI	5.36 ± 6.51	64.9 ± 25.49	40.1 ± 23.28
TBUT (seconds)	12.17 ± 1.71	2.39 ± 0.67	5.63 ± 0.66
Meibography grading	0 ± 0.0	1 ± 0.0 (1-1)	0.5 ± 0.58

Table 7.1 Profiles of the normal subjects consented for this study

7.3.2 Equipment

The controlled environment chamber (CEC) was employed for the two visits made by each subject, on separate days. It was decided randomly, on the toss of a coin, whether the subject would receive saline or anaesthetic drops on his or her first visit. Tear osmolarity was routinely measured in all subjects prior to entry into the CEC, in uncontrolled clinic conditions, in the area immediately outside the chamber. Room temperature and RH were recorded at the time of these measurements (Table 7.3). Experiments were conducted in controlled, 'desiccating' environment conditions of 5% RH, temperature 23°C, and airflow 0.08 m/s (Table 7.2). A 10 minute period was required after the subject and examiner had entered the CEC to allow for equilibration of the environmental conditions. Tear osmolarity was measured using the TearLab® osmometer. Corneal anaesthesia was achieved using topical anaesthetic eye drops, proxymetacaine 0.5% and tetracaine 1%, and saline was used in the control studies (see Chapters 2 and 3 for details). To minimise reflex tearing, a single drop of the short-acting anaesthetic 0.5% proxymetacaine (Minims®) (Jones, 1966) was instilled first, followed 30 seconds later by a drop of 1% tetracaine (Minims®). It was assumed that a deep to moderately deep anaesthesia would last approximately 30 minutes with a lesser degree of corneal anaesthesia remaining for up to one hour. The protocol was repeated with the saline eye drops serving as a control.

	Temperature	Relative Humidity	Airflow
'Desiccating conditions'	23°C	5%	0.08m/s
'Clinic conditions'	Uncontrolled environment: temperature and relative humidity recorded at each visit		

Table 7.2 Environmental conditions employed in this study.

7.3.3 Protocol

The saline control visit lasted approximately one hour; the subject and the examiner entered the CEC and at zero time one drop of saline (control) was instilled into each eye, followed by another at 30 seconds (to control for the anaesthetic studies). Excess tears were dabbed away with tissues by the investigator and the subjects remained with their eyes open, blinking spontaneously to facilitate tear drainage. Tear osmolarity was measured first in the right eye followed by the left eye every 20 minutes for one hour, during which time the subject was instructed to keep the eyes open looking straight ahead and blinking spontaneously.

The topical anaesthesia visit lasted approximately one hour and took place on a different day, but at a similar time of day. The subject and the examiner entered the CEC and at zero time, one drop of proxymetacaine 0.5% was instilled into each eye followed after 30 seconds by one drop of tetracaine 1% into each eye. Excess tears were dabbed away with tissues by the investigator and the subjects remained with their eyes open, blinking spontaneously to facilitate tear drainage. Tear osmolarity was measured first in the right eye, then the left eye, every 20 minutes for one hour, during which time the subject was instructed to keep the eyes open looking straight ahead and blinking spontaneously.

A slit-lamp examination was carried out following each visit to check for adverse events. The Oxford scale was used to grade any corneal staining present following exposure to desiccating conditions on a scale from 0-5 (See Figure 7.1 for a timeline of the experiments and Appendix XI for an example record card).

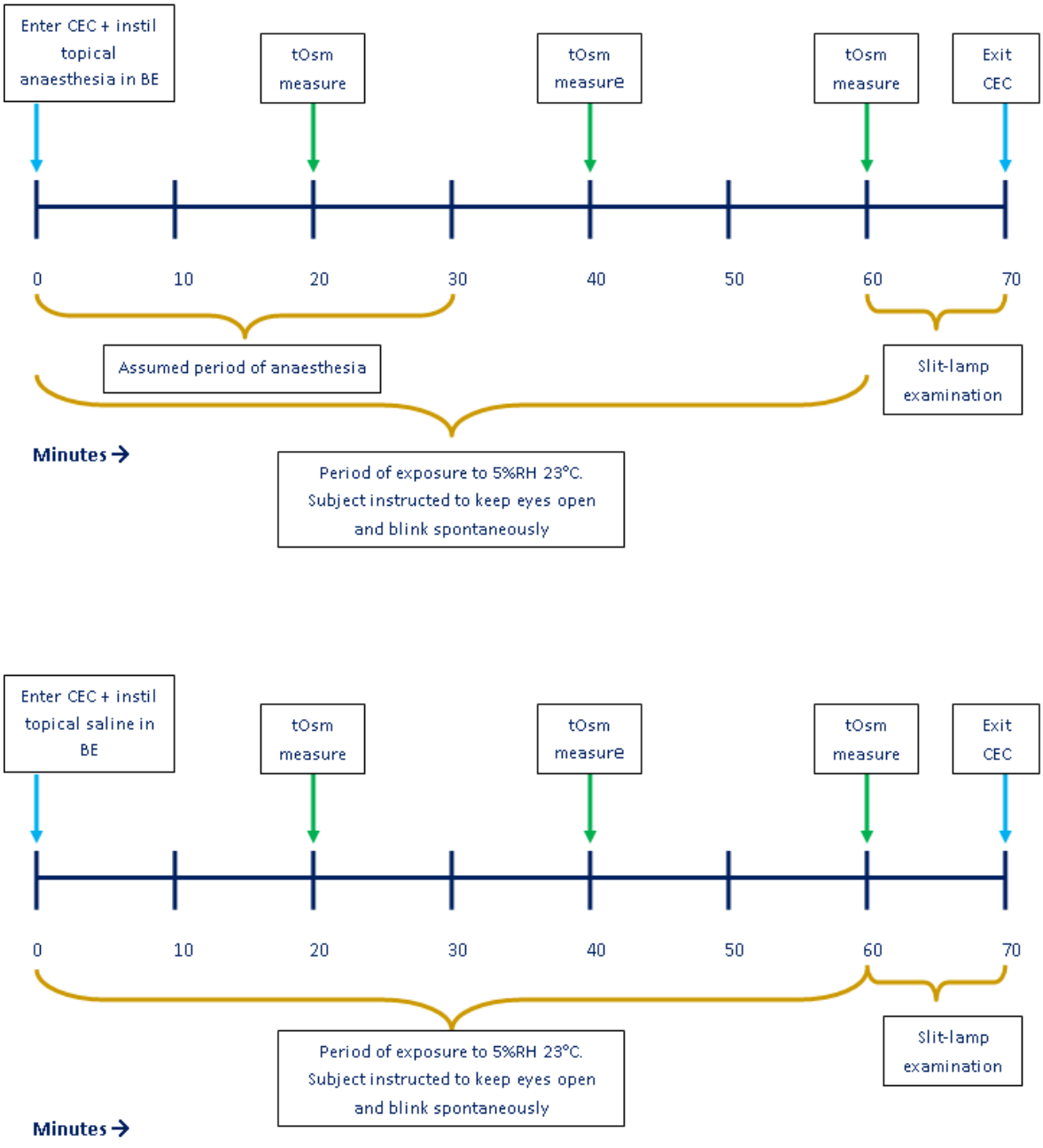


Figure 7.1 Timeline of anaesthetic and saline visits.

7.3.4 Statistical analysis

A non-parametric Friedman test (due to outliers, indicated in the Tables 7.4-7.7) was used to analyse the mean of tOsm values, which were compared across the 3 time points in the CEC and with the value measured in clinic conditions; the values were analysed in the right eye (RE) and left eye (LE), in normal and DED subjects and in the saline and anaesthesia conditions. An exception to this pathway of analysis was the data for DED in the LE following saline instillation; in this instance there were no outliers and the data was normally distributed at each time point, and a one-way repeated measures ANOVA test

was used. A related samples sign test was used to calculate the difference in corneal staining graded with the Oxford staining scale, before and after exposure to desiccating conditions and following saline or anaesthetic instillation; the right eye in each subject was used for analysis and presented in this chapter. The Bonferroni method was used to make adjustments for multiple comparisons. Shapiro-Wilk test was used to evaluate normality of distribution. Differences were considered statistically significant with *P* values less than 0.05. Data is presented as mean ± standard deviation. Analyses were performed using SPSS 20.

7.4 RESULTS

Details of the uncontrolled clinic conditions for all 32 experiments (16 from the experiments with normal subjects and 16 from the DED patients) are presented in Table 7.3. The temperature ranged from 18°C to 27°C and the RH ranged from 26% to 60%. These measures were recorded to show the variation encountered in a clinical environment on a day-to-day basis in comparison to the controlled environment created in the CEC.

Experiment number	**Temperature (°C)**		**Relative Humidity (%)**	
	Normal	**DED**	**Normal**	**DED**
1	23	22	50	43
2	18	26	42	35
3	23	27	50	39
4	18	24	42	33
5	22	22.8	38	45
6	24	22.9	37	44
7	21	22.4	45	54
8	26	22.8	37	52
9	21	21.6	45	45
10	26	22.6	36	51
11	27	22	37	40
12	26	21	42	60
13	22	23	44	47
14	22.2	22.5	26	49
15	22	23	41	40
16	22	22.7	49	54
Range	**9.0**	**6.0**	**24.0**	**27.0**
Min/Max	**18°C to 27°C**	**21°C to 27°C**	**26% to 50%**	**33% to 60%**

Table 7.3 Temperature and RH levels recorded in the uncontrolled clinic conditions.

Normal subjects - topical anaesthetic

The tOsm data for both eyes in normal subjects are presented in Table 7.4 and Figure 7.2. The mean RE tOsm started at 297.1 ± 5.67 mOsm/L in clinic conditions, decreased to 293.4 ± 7.93 after 20 minutes exposure to 5% RH, 293.5 ± 7.67 after 40 minutes exposure and was at 292.0 ± 7.07 after 60 minutes. The differences in tOsm across the time points were not statistically significant: $\chi^2(3) = 3.627$, $p = 0.305$. In the LE tOsm started at 298.6 ± 7.78 mOsm/L in clinic conditions, decreased to 296.5 ± 6.35 after 20 minutes exposure, 295.4 ± 7.80 after 40 minutes exposure and was at 291.8 ± 6.98 after 60 minutes. The differences were not statistically significant: $\chi^2(3) = 4.948$, $p = 0.176$.

Subject	**tOsm @ clinic conditions**		**tOsm @ 20 minutes**		**tOsm @ 40 minutes**		**tOsm @ 60 minutes**	
	R	**L**	R	**L**	R	**L**	R	**L**
1	300	**289**	309	**283**	279∞	**303**	280∞	**279∞**
2	305	**307**	296	**300**	290	**294**	290	**293**
3	292	**299**	289	**300**	293	**296**	292	**293**
4	299	**289**	289	**301**	301	**291**	293	**294**
5	304	**311**	*	**295**	294	**310**	301	**302**
6	294	**301**	293	**292**	293	**293**	290	**293**
7	290	**297**	300	**301**	293	**285**	288	**285**
8	293	**296**	306	**300**	305	**291**	302∞	**296**
Mean ± SD	297.1 ± 5.67	**298.6 ± 7.78**	293.4 ± 7.93	**296.5 ± 6.35**	293.5 ± 7.67	**295.4 ± 7.80**	292.0 ± 7.07	**291.8 ± 6.98**

* Value discarded due to sampling problems

∞ Data outlier

Table 7.4 Data for normal subjects in 'clinic conditions' and at three time points with topical anaesthetic instillation and exposure to 'desiccating conditions'.

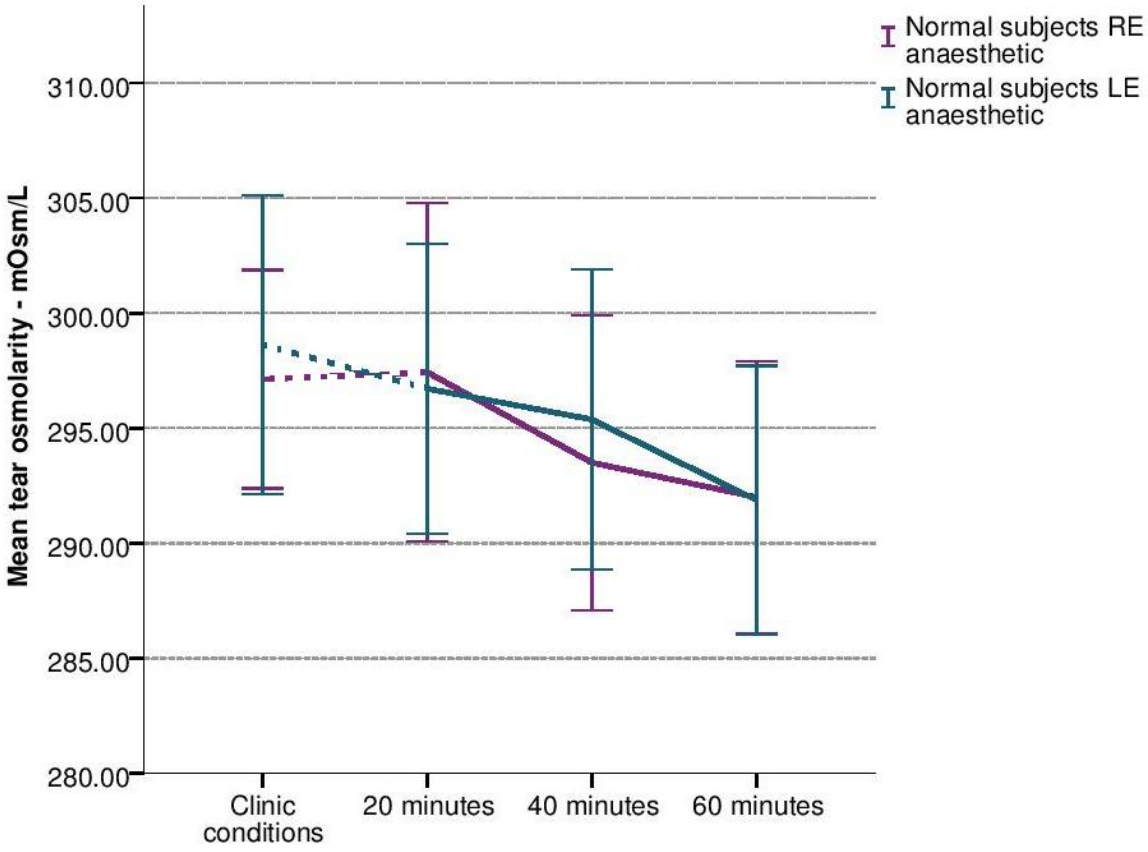


Figure 7.2 Tear osmolarity in normal subjects following instillation of topical anaesthetic in clinic conditions and at 20 minutes time points during exposure to 'desiccating conditions' for 60 minutes.

Normal subjects - saline

The tOsm data for both eyes in normal subjects are presented in Table 7.5 and Figure 7.3. In the RE tOsm started at 300.5 ± 5.98 mOsm/L in clinic conditions, decreased to 295.1 ± 3.13 after 20 minutes exposure, increased again to 299.0 ± 7.37 after 40 minutes exposure and was at 291.4 ± 5.44 after 60 minutes exposure to desiccating conditions. The differences across the time points were not statistically significant: $\chi^2(3) = 5.644$, $p = 0.130$. In the LE tOsm started at 299.1 ± 8.08 mOsm/L in clinic conditions, decreased to 292.6 ± 5.50 after 20 minutes exposure, increased again to 296.1 ± 4.52 after 40 minutes exposure and was at 297.1 ± 9.98 after 60 minutes. The differences were not statistically significant: $\chi^2(3) = 3.987$, $p = 0.263$.

Subject	tOsm @ clinic conditions		tOsm @ 20 minutes		tOsm @ 40 minutes		tOsm @ 60 minutes	
	R	**L**	R	**L**	R	**L**	R	**L**
1	301	**289**	291	**287**	*	**301**	283	**293**
2	297	**292**	295	**294**	297	**297**	292	**293**
3	294	**299**	293	**284**	299	**293**	297	**287**
4	298	**299**	300	**296**	302	**299**	299	**301**
5	298	**300**	293	**291**	299	**293**	292	**290**
6	314	**316**	*	**302**	311	**301**	*	**317**
7	301	**302**	296	**294**	286∞	**297**	289	**305**
8	301	**296**	298	**293**	299	**288**	288	**291**
Mean ± SD	300.5 ± 5.98	**299.1 ± 8.08**	295.1 ± 3.13	**292.6 ± 5.50**	299.0 ± 7.37	**296.1 ± 4.52**	291.4 ± 5.44	**297.1 ± 9.98**

* Value discarded due to sampling problems

∞ Data outlier

Table 7.5 Data for normal subjects in 'clinic conditions' and at three time points with saline (control) instillation and exposure to 'desiccating conditions'.

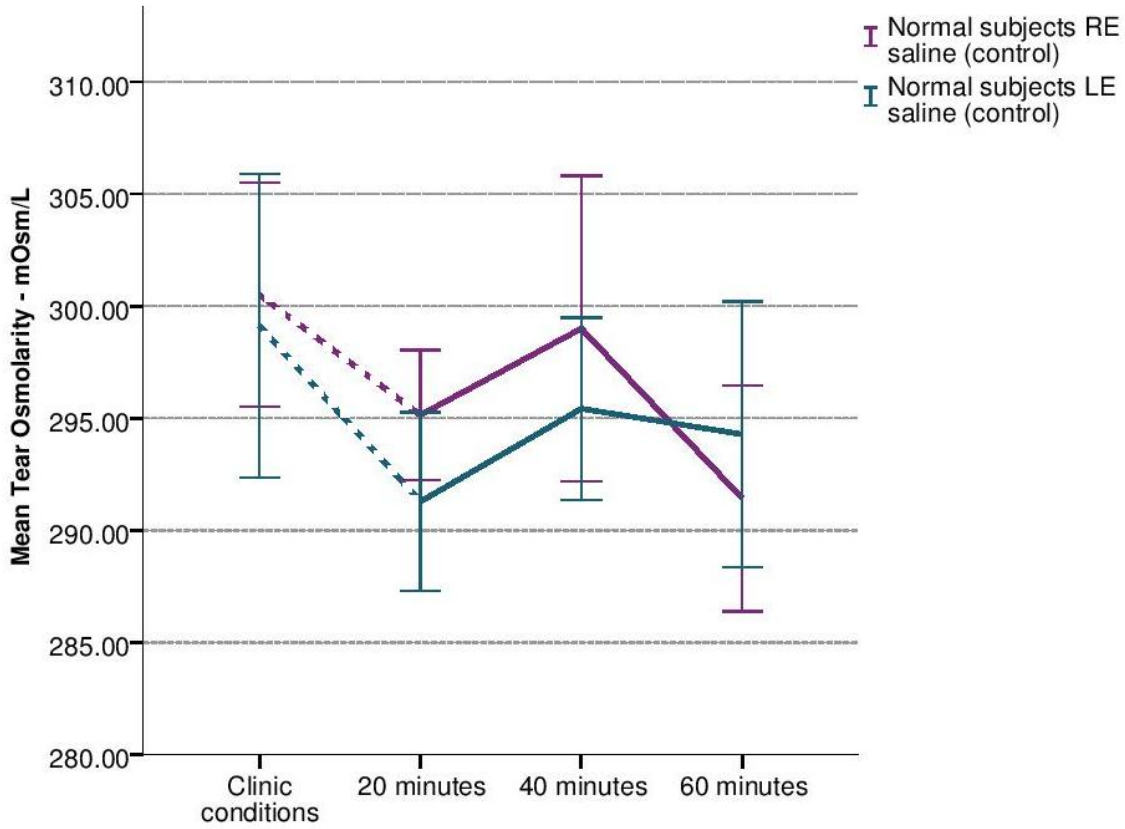


Figure 7.3 Tear osmolarity in normal subjects following instillation of topical saline in clinic conditions and at 20 minutes time points during exposure to 'desiccating conditions' for 60 minutes.

DED Subjects - topical anaesthetic

The tOsm data for the right and left eyes in DED patients are presented in Table 7.6 and Figure 7.4. In the RE tOsm started at 298.3 ± 13.67 mOsm/L in clinic conditions, decreased to 294.5 ± 16.19 after 20 minutes exposure, 293.5 ± 14.35 after 40 minutes exposure and was at 295.9 ± 14.57 after 60 minutes exposure to desiccation. The differences across the time points were not statistically significant: $\chi^2(3) = 3.329$, $p = 0.344$. In the LE tOsm started at 298.9 ± 6.29 mOsm/L in clinic conditions, decreased to 293.4 ± 11.34 after 20 minutes exposure, increased to 296.4 ± 12.70 after 40 minutes exposure and was at 292.8 ± 9.11 after 60 minutes. The differences were not statistically significant: $\chi^2(3) = 0.846$, $p = 0.838$.

Subject	**tOsm @ clinic conditions**		**tOsm @ 20 minutes**		**tOsm @ 40 minutes**		**tOsm @ 60 minutes**	
	R	**L**	R	**L**	R	**L**	R	**L**
1	313	**298**	298	**298**	298	**317**	306	**299**
2	296	**295**	287	**302**	297	**296**	296	**297**
3 ~	299	**297**	296	**281**	293	**284**	294	**284**
4 ~	300	**302**	285	**283**	280	**282**	276	**281**
5 ~	277	**300**	331∞	**306**	323∞	**309**	321	**301**
6 ~	282	**288**	282	**282**	283	**284**	282	**281**
7	302	**301**	281	**308**	278	**297**	287	**302**
8	317	**310∞**	296	**287**	296	**302**	305	**297**
Mean ± SD	298.3 ± 13.67	**298.9 ± 6.29**	294.5 ± 16.19	**293.4 ± 11.34**	293.5 ± 14.35	**296.4 ± 12.70**	295.9 ± 14.57	**292.8 ± 9.11**

∞ Data outlier

~ Subject diagnosed with Sjogren syndrome

Table 7.6 Data for DED patients in 'clinic conditions' and at three time points with topical anaesthetic instillation and exposure to 'desiccating conditions'.

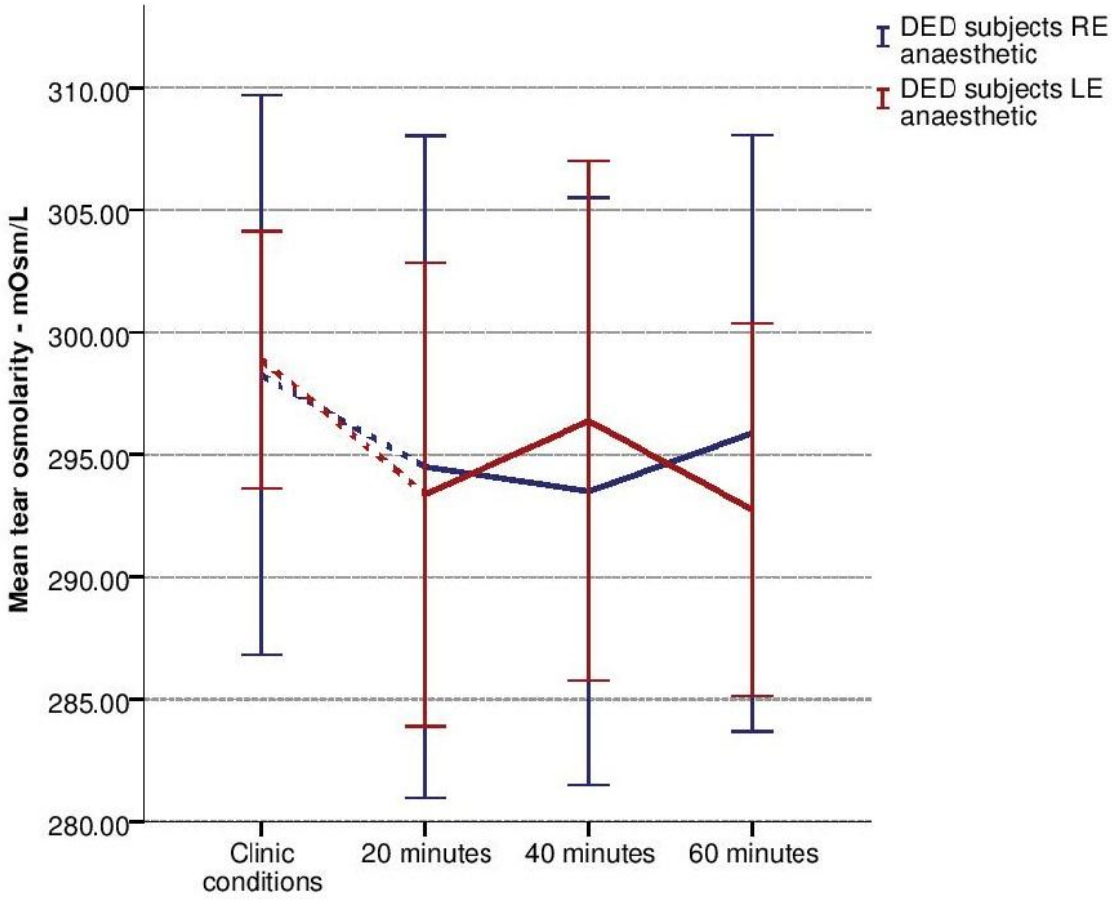


Figure 7.4 Tear osmolarity in DED subjects following instillation of topical anaesthetic in clinic conditions and at 20 minutes time points during exposure to 'desiccating conditions' for 60 minutes.

DED subjects - saline

The tOsm data for both eyes in DED patients are presented in Table 7.7 and Figure 7.5. In the RE tOsm started at 296.6 ± 13.85 mOsm/L in clinic conditions, increased to 304.3 ± 15.14 after 20 minutes exposure, decreased to 297.5 ± 9.58 after 40 minutes exposure and was at 297.0 ± 10.01 after 60 minutes of exposure to desiccation. The differences across the time points were not statistically significant: $\chi^2(3) = 2.338$, $p = 0.505$. In the LE tOsm started at 298.0 ± 6.14 mOsm/L in clinic conditions, increased to 298.8 ± 8.94 after 20 minutes exposure, increased again to 300.4 ± 8.94 after 40 minutes exposure and was at 293.9 ± 7.85 after 60 minutes. The differences were not statistically significant: $F_{(1.689, 11.824)} = 1.499$, $p = 0.260$.

Subject	tOsm @ clinic conditions		tOsm @ 20 minutes		tOsm @ 40 minutes		tOsm @ 60 minutes	
	R	**L**	R	**L**	R	**L**	R	**L**
1	312	**309**	321	**307**	319∞	**317**	314	**290**
2	290	**298**	298	**300**	299	**301**	306	**300**
3 ~	292	**296**	292	**288**	289	**309**	286	**291**
4 ~	282	**294**	302	**301**	292	**292**	286	**293**
5 ~	312	**297**	329	**298**	292	**291**	296	**286**
6 ~	290	**290**	285	**286**	292	**295**	290	**284**
7	281	**295**	295	**297**	296	**302**	295	**300**
8	314	**305**	312	**313**	301	**296**	303	**307**
Mean ± SD	296.6 ± 13.85	**298.0 ± 6.14**	304.3 ± 15.14	**298.8 ± 8.94**	297.5 ± 9.58	**300.4 ± 8.94**	297.0 ± 10.01	**293.9 ± 7.85**

∞ Data outlier

~ Subject diagnosed with Sjögren syndrome

Table 7.7 Data for DED patients in 'clinic conditions' and at three time points with saline (control) instillation and exposure to 'desiccating conditions'.

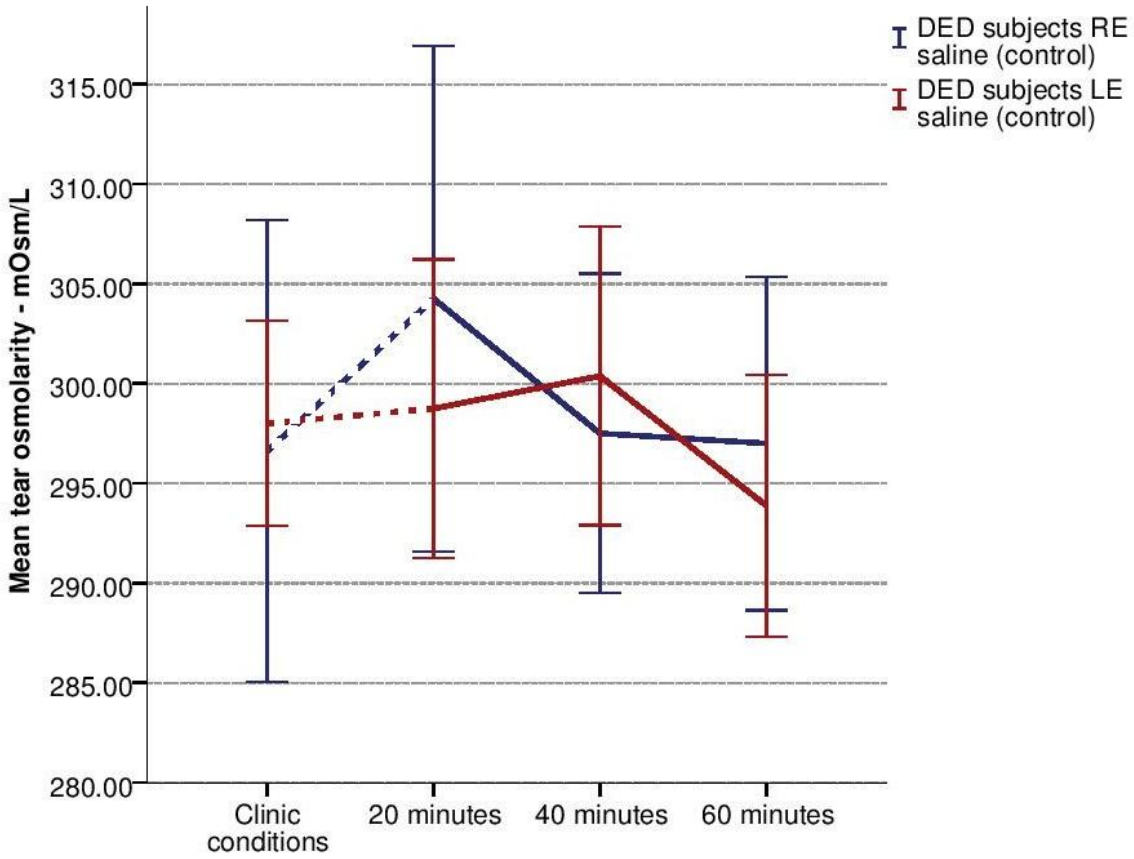


Figure 7.5 Tear osmolarity in DED subjects following instillation of topical saline in clinic conditions and at 20 minutes time points during exposure to 'desiccating conditions' for 60 minutes.

A slit-lamp examination was carried out after each visit in the CEC to monitor for any adverse effects of exposure to the low RH environment; the Oxford grading scale was used to grade any corneal staining present pre and post exposure to desiccating

conditions (Appendix V). The right eye of each subject in the anaesthetic study was used for analysis and presented here.

Normal group

The mean corneal staining grade in the normal group pre-exposure was 1.25 ± 1.16. After 60 minutes exposure to desiccating conditions and saline instillation the mean corneal staining grade was 1.50 ± 1.19 and after 60 minutes exposure to desiccating conditions and instillation of anaesthetics the mean corneal staining was 1.50 ± 1.19. Neither post-exposure corneal staining values reached significance when compared to the pre-exposure level (p = 0.50). Figures 7.6 and 7.7 show images for the eight subjects demonstrating the pre-exposed cornea staining levels and post-exposure to desiccating stress plus anaesthetic instillation corneal appearance of the right eye.

DED group

The mean corneal staining grade in the DED group pre exposure was 2.63 ± 3.50. After 60 minutes exposure to desiccating conditions and saline instillation the mean corneal grading was 2.75 ± 3.46; this difference did not reach significance compared to the pre exposure level (p = 0.50). Following instillation of anaesthetics and 60 minute exposure to desiccating conditions the mean corneal staining grade was 4.63 ± 3.96, which was significantly increased when compared to the level at pre-exposure level (p = 0.031) and was observed particularly in the inferior portion of the cornea, and more profoundly in the DED patients diagnosed with Sjögren syndrome. Figures 7.8 and 7.9 show images for the eight subjects demonstrating the corneal appearances pre-exposure cornea staining levels and post-exposure to desiccating stress plus anaesthetic instillation of the right eye.

Subjects who displayed corneal staining of over Grade 1 were instructed to return on the next day for a further corneal examination, at which time it was observed in every case that the corneal appearance had returned to the pre-exposure level. Images of the left cornea following anaesthetic instillation and of both eyes following saline instillation in both the normal and DED groups are shown in Appendix XIII.

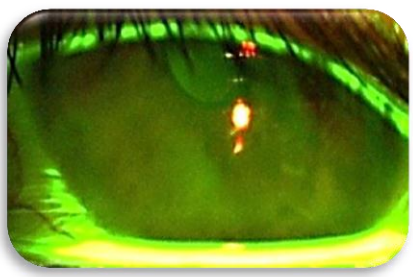
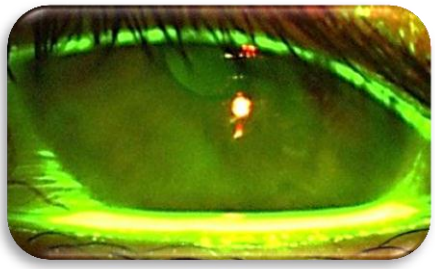
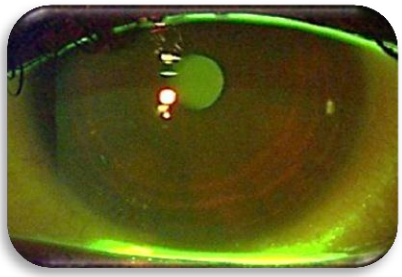
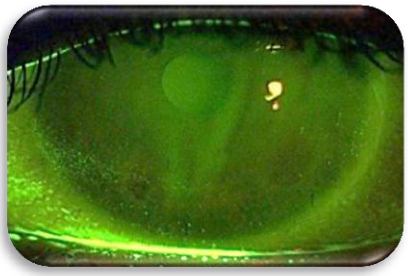
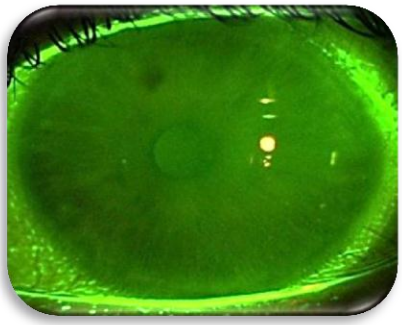
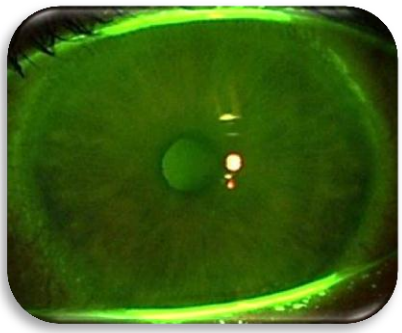
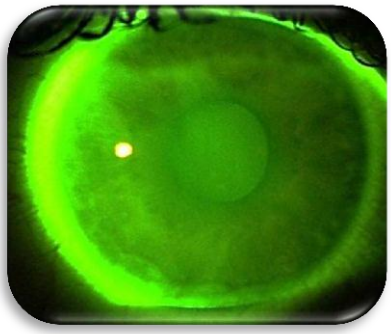
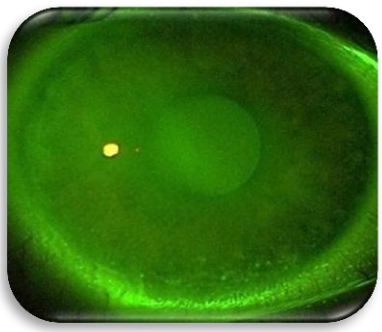
Subject 1 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 2 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 3 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 4 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**

Figure 7.6 Slit-lamp images with fluorescein pre and post CEC exposure to desiccating conditions for 60 minutes and after anaesthetic instillation in right eyes of normal subjects 1-4.

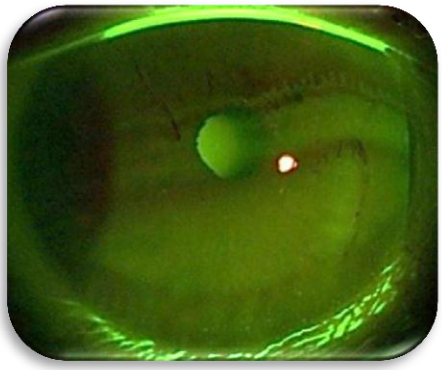
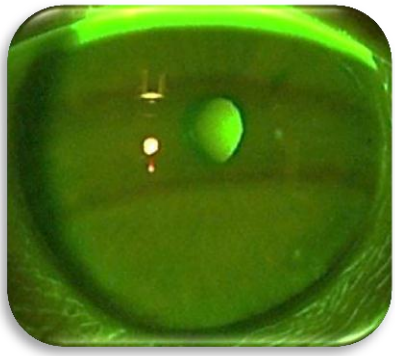
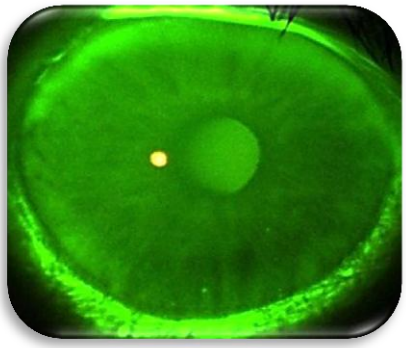
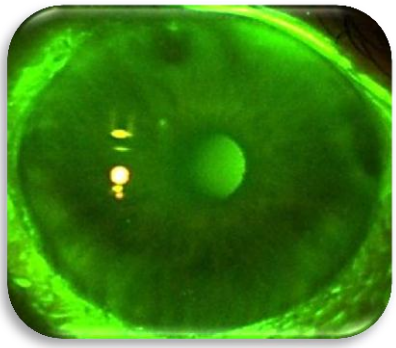
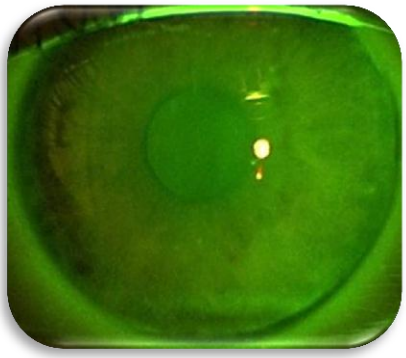
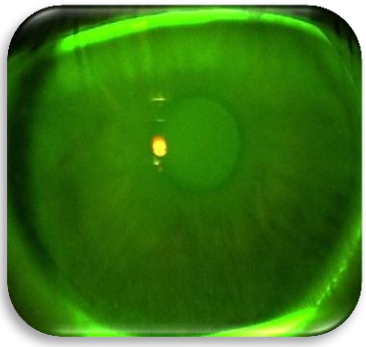
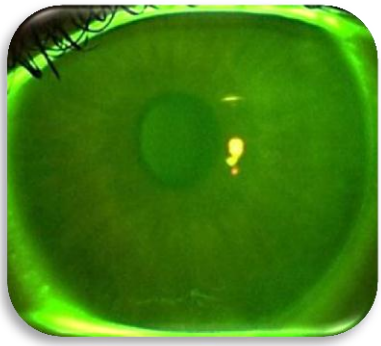
Subject 5 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 6 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 7 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 8 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**

Figure 7.7 Slit-lamp images with fluorescein pre and post CEC exposure to desiccating conditions for 60 minutes and after anaesthetic instillation in right eyes of normal subjects 5-8.

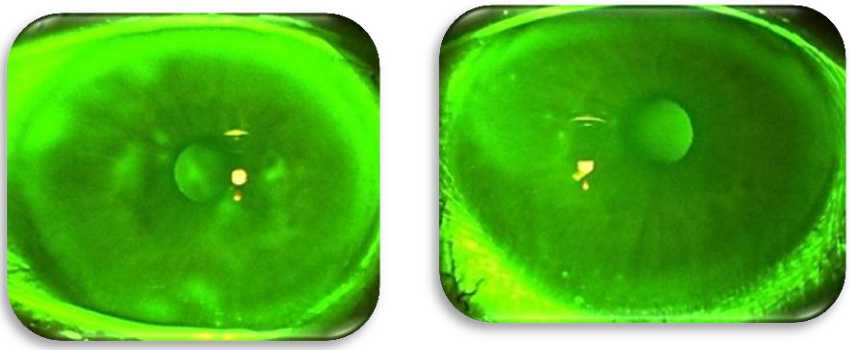
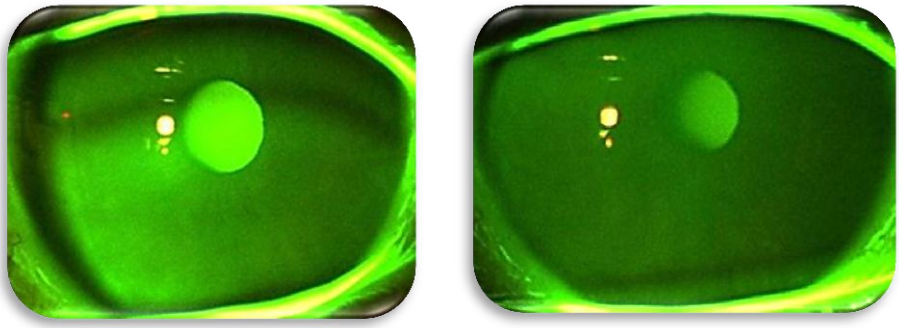
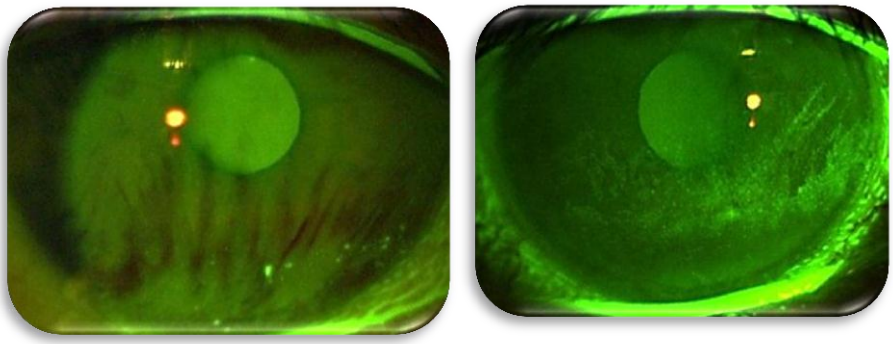
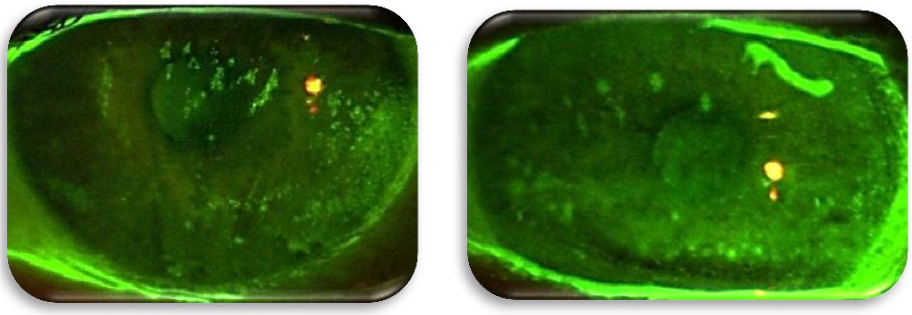
Subject 1 **DED**	 **PRE-EXPOSURE** **POST-EXPOSURE**
Subject 2 **DED**	 **PRE-EXPOSURE** **POST-EXPOSURE**
Subject 3 **DED (SjS)**	 **PRE-EXPOSURE** **POST-EXPOSURE**
Subject 4 **DED (SjS)**	 **PRE-EXPOSURE** **POST-EXPOSURE**

Figure7.8 Slit-lamp images with fluorescein pre and post CEC exposure to desiccating conditions for 60 minutes and after anaesthetic instillation in right eyes of DED patients 1-4.

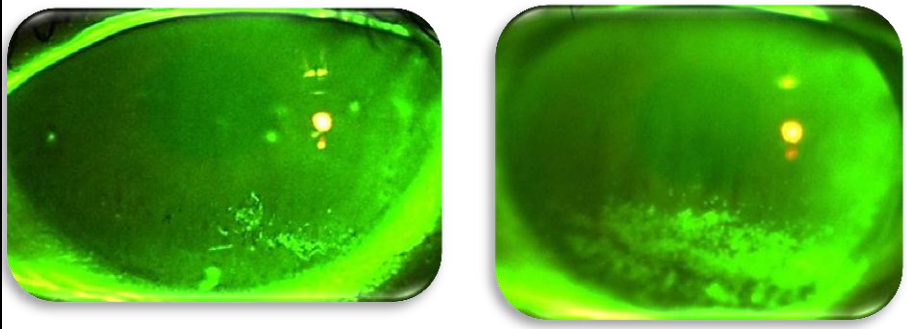
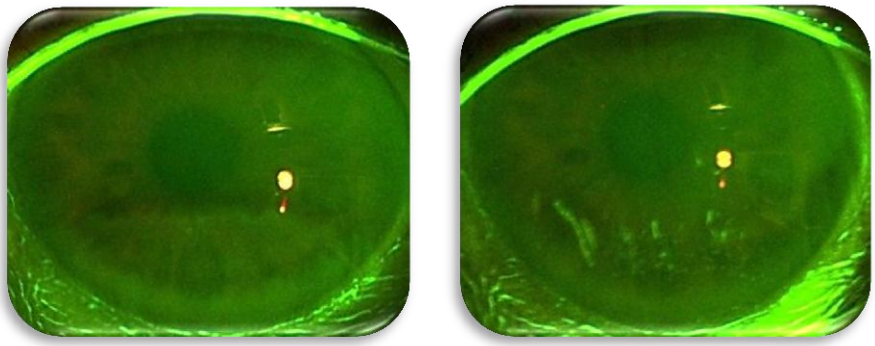
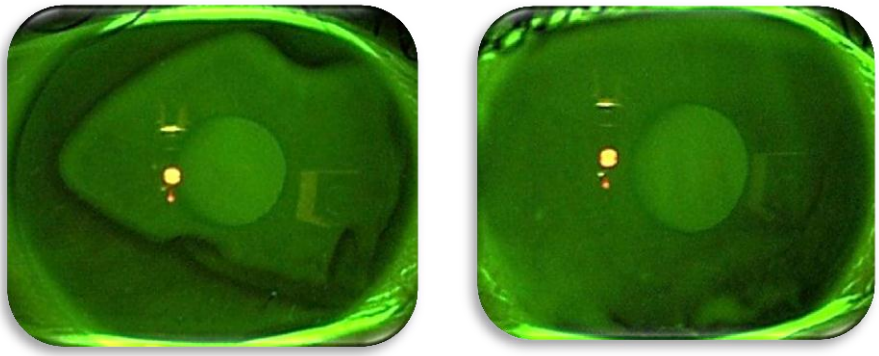
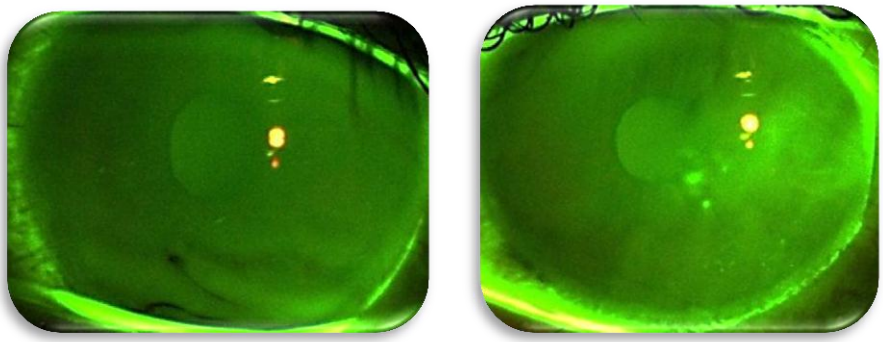
Subject 5 **DED (SjS)**		**PRE-EXPOSURE** **POST-EXPOSURE**
Subject 6 **DED (SjS)**		**PRE-EXPOSURE** **POST-EXPOSURE**
Subject 7 **DED**		**PRE-EXPOSURE** **POST-EXPOSURE**
Subject 8 **DED**		**PRE-EXPOSURE** **POST-EXPOSURE**

Figure 7.9 Slit-lamp images with fluorescein pre and post CEC exposure to desiccating conditions for 60 minutes and after anaesthetic instillation in right eyes of DED patients 5-8.

7.5 DISCUSSION

Exposure to desiccating conditions, especially low relative humidity has been found to increase symptoms and signs of dry eye disease (DED) (Tsubota and Yamada, 1992; Tsubota, 1998; Uchiyama et al., 2007). In a healthy eye hyperosmolarity triggers an afferent sensory response from the ocular surface that, via the lacrimal functional unit (LFU) stimulates a compensatory reflex tearing, which re-establishes homeostasis. In DED the system that allows the compensatory mechanism to function becomes increasingly faulty, and subsequently a prolonged state of hyperosmolarity may compromise the corneal sensitivity and hence reduce afferent signals. A positive correlation between loss of sensation and the degree of ocular surface staining and a negative correlation between surface damage and symptoms, suggests a progressive increase in sensory loss with disease progression (Adatia et al., 2004).

This is the first experiment to use topical anaesthetics, theorised to temporarily block the afferent signals from the cornea with the result that in conditions of desiccating stress the LFU could not respond, and to include DED subjects in the protocol. It was hypothesised that this would lead to a deficiency in compensatory tearing and a consequent rise in tear osmolarity (tOsm), the effect being more pronounced in DED than in normal subjects. The experiment using saline eye drops was designed to serve as a control for the use of anaesthetic drops. It was expected that, following the instillation of topical saline, tOsm would not change significantly over the period of an hour exposed to desiccating conditions, since a normally functioning LFU would be expected to compensate for the adverse conditions.

There was no significant increase in the tOsm from pre-exposure levels to that measured over the course of one hour exposure to desiccating conditions in the control experiment (saline), in either eye of the normal group (RE $p = 0.130$; LE $p = 0.263$) or the DED group (RE $p = 0.505$; LE $p = 0.260$). Unexpectedly, the tOsm pre and post exposure following anaesthetic instillation also did not show a significant increase in tOsm in either eye of the normal group (RE $p = 0.305$; LE $p = 0.176$) or in either eye of the DED group (RE $p = 0.344$; LE $p = 0.838$). Although these findings are in line with those of previous workers involving exposure to desiccating conditions (Abusharha and Pearce, 2013; Teson et al., 2013; López-Miguel et al., 2014), it is noted that in those studies, topical anaesthesia was not employed.

A lack of significant change in tOsm here after anaesthetic use and exposure to desiccation could be due to many factors, most likely due to the small numbers of subjects recruited into the study but also the possibility that the desiccating stress created in the CEC was not sufficient to elicit a tOsm response. Previous pilot studies (Chapter 3) had

indicated that adding a level of increased airflow for 5 minutes, induced significant corneal response and resulted in grade 5 corneal staining; as a result this was not incorporated into the final protocol presented here. More likely, the period of topical anaesthesia was insufficient to permit the loss of compensatory response to influence osmolarity. Blink rate was not recorded during the study and may have increased, which would have had a protective effect. An additional factor could have been that clearance of instilled anaesthetic drops was inadequate so that they had a diluting effect on the tears, or that the level of reflex tear response to the instilled proxymetacaine was greater than anticipated. Additionally, the osmolarity of the instilled drops was unknown and could have influenced the final measured tOsm result. The expectation that an effect, if it occurred, would be amplified in the DED patients exposed to anaesthetic was not realised and it may be noted that, despite recruitment of all DED patients at a tOsm of ≥308 mOsm/L or more, their tOsms on the days of the experiments were rarely at or above these levels, implying that disease severity was mild.

An approach to circumvent the problems cited above could be to recruit patients with dense unilateral anaesthesia, as in neurotrophic keratitis (NK), to the study. In this case bilateral anaesthesia could be achieved by the instillation of topical anaesthetic into the normal fellow eye, and anaesthesia in this fellow eye could be topped up during the period of exposure to desiccating stress without inducing reflex tearing. In this way it might be possible to extend the period of observation under full anaesthesia, in the NK eye, to an hour and problems of diluting tears by instilling drops into the experimental eye would be avoided.

Sensory inputs from the nasal mucosa could also play a role in the compensatory effects observed here. Studies by Heigle and Pflugfleder (1996) and Gupta et al., (1997) have demonstrated that anaesthesia of the nasal mucosa resulted in a decrease in lacrimal secretion measured by the Schirmer test (Heigle and Pflugfelder, 1996; Gupta, Heigle and Pflugfelder, 1997). In the case of the subjects here, an assumed functioning sensory input from this area could have been sufficient to offset the lack of input from the ocular surface and stimulate lacrimal secretion. Studies in animals (Brinton et al., 2015; Kossler et al., 2015) and in humans (Friedman et al., 2016; Gumus and Pflugfelder, 2017) have reinforced the idea that there is a reflex link to lacrimal secretion from the nasal mucosa. The intranasal tear stimulator (Allergan Plc, Dublin, Ireland) that has been developed applies an electrical stimulus to the trigeminal sensory nerves in the nasal cavities, which initiates the afferent limb of the nasolacrimal reflex response. Friedman et al., (2016) measured the effect of using this device for three minutes on 40 mild to severe DED patients (Friedman et al., 2016). The group reported a Schirmer wetting of 9.4 ± 1.3mm unstimulated and 22.4 ± 1.9mm stimulated in the right eye and 10.5 ± 1.1mm

unstimulated and 22.5 ± 1.8mm stimulated in the left eye. This represented an increase in aqueous tear production of 138.3% and 114.3% in the right and left eye respectively.

Following exposure in the CEC a slit-lamp examination was carried out routinely to check for adverse events. In the normal group there was no significant change in corneal staining at recruitment (pre-exposure) compared to that recorded following exposure to desiccating conditions and instillation of either saline or anaesthetic drops (p = 0.50). No significant change in corneal staining was also observed in the DED group from the pre-exposure recruitment stage compared to exposure and instillation of saline eye drops (p = 0.50). However, in the DED group the corneal staining observed at recruitment was measured at 2.63 ± 3.50 and following exposure to desiccating conditions and instillation of anaesthetic eye drops increased to 4.63 ± 3.96 which reached significance (p = 0.031). The corneal staining was observed mainly in the inferior portion of the cornea and primarily in the DED patients who suffered from Sjögren syndrome (see Figures 7.8 and 7.9). These subjects did present with a higher level of corneal staining prior to exposure but as a likely consequence of an inherently reduced aqueous volume caused by their underlying disease this damage was compounded. Sjögren syndrome patients have also been shown to have a higher prevalence of meibomian gland dysfunction (MGD), reported as 57.9% in the Sjögren group versus 18.5% in a normal group (Shimazaki et al., 1998) which could have contributed the effect due to a higher evaporation from the ocular surface, although in the present study the patients recruited did not have significant MGD. Incomplete blinking could explain why the staining following exposure to the desiccating conditions occurred more readily in the inferior portion of the cornea since this appears to encourage thinning of the pre ocular tear film in an area that is not refreshed by the tear film after an incomplete blink, enhancing the opportunity for further evaporation. It may also follow that subsequent to the incomplete blink event, if a full blink is attempted this may increase the insult to the corneal surface via friction (McMonnies, 2007). All subjects exhibiting corneal staining of grade >1 were re-examined the next day, when the corneal staining was found to have resolved or reduced to baseline levels within this 24 hour period, which is in line with previous studies that have documented punctate staining to be transient, appearing and disappearing over a matter of hours (Caffery and Josephson, 1991; Garofalo et al., 2005).

The findings of this experiment did not elicit the results predicted, since there was no significant rise in tOsm in either group of subjects after one hour exposure to 5% RH with sensory blockade. This could have been due to a number of the factors discussed earlier. However, the presence of corneal staining following this exposure and sensory blockade in the DED group did indicate that a change was occurring at the ocular surface, which was not reflected in the osmolarity of the tears sampled in the meniscus. It has previously been indicated by modelling considerations that the tOsm measured at the level of the

meniscus does not necessarily reflect that occurring across the whole corneal surface, the tear film osmolarity exceeding that of the meniscus and being more pronounced in DED subjects (Gaffney et al., 2010). Thus it may be hypothesised that in the current studies, in the DED patients desiccating stress caused tOsm to rise to a damaging level locally, but that the magnitude of this effect was not sufficient to influence the bulk of the tears or at least that portion sampled from the meniscus. This could also be the reason for the often reported discordance between signs and symptoms when related to DED and could have implications for the monitoring of individual responses to treatment on the basis of tOsm (Bartlett et al., 2015). In other studies the highest correlation between signs and symptoms was found between staining in the inferior portion of the cornea and evening intensity of discomfort and dryness (Begley et al., 2003; Rodriguez et al., 2016).

To conclude, it would be of great use to be able to sample the tear film from the surface of the eye directly to gain a fuller picture of the tOsm levels at a tissue level, and since these experiments were conducted, a new device that measures tOsm at the level of the conjunctiva, the i-Pen®, has become available. This device measures tOsm of the tears from a sample taken from the palpebral conjunctiva, and reported to reflect the osmolarity of the tissue at this site, although recent reports differ as to the accuracy of the instrument (Reis, Greiner and Albuquerque, 2017; Rocha et al., 2017).

Chapter 8:

General Conclusions and Future Work

Tear Dynamics relates to the exquisitely sensitive homeostatic system that maintains a steady state at the ocular surface, ensuring stability of the tear film despite an array of potentially disturbing, internal and external influences. In dry eye disease (DED) this homeostatic mechanism is disrupted and the resulting tear hyperosmolarity leads to ocular surface damage and debilitating ocular surface symptoms (Lemp et al., DEWS I 2007; Craig et al., 2017). The lacrimal functional unit (LFU), responsible for compensatory tear production and adjustments to the blink rate, can mitigate the effects of adverse environments (Tsubota and Nakamori, 1995; Stern et al., 2004). When the eyes are open and the ocular surface is exposed to a variety of stresses (low humidity, wind, cold and other physical or chemical agents), the reflex pathway adjusts lacrimal secretion and blink rate to achieve osmolar homeostasis. In conditions of high humidity, equable temperature, zero airflow and absent chemical exposure, the secretion will be low and may be considered to be 'basal'. In conditions of desiccating stress, sensory drive is predicted to rise in proportion to the severity of the conditions and results in increased lacrimal secretion, working by activation of the LFU.

In the experiments presented here a controlled environment chamber (CEC) was utilised to simulate a variety of different ambient conditions e.g. standard/office room conditions, conditions of evaporative suppression and conditions of desiccating stress. The research investigated the LFU responses to adverse environmental conditions, in particular tear physiology and its relationship to DED, using either tear osmolarity (tOsm) or production (measured by the Schirmer test) as outcome measures to represent changes in functioning.

The first experiments (Chapter 4) studied the influence of desiccation on Schirmer test results. The Schirmer test has long been used as a diagnostic tool for DED; however, many studies have reported poor reproducibility and high levels of variability in this test (Kallarackal et al., 2002; Loran et al., 1987; Serin et al., 2007; Sullivan et al., 2010). Holly et al., (1984) showed that the wetting length of the Schirmer strip was influenced by evaporation and would therefore be affected by ambient humidity (Holly, Laukaitis and Esquivel, 1984). The experiments in this chapter explored whether exposure to desiccating stress would result in a falsely low Schirmer value. It was hypothesised that by preventing evaporation from the strip, the response would be freed from the influence of ambient conditions, of particular importance in a desiccating environment. Normal

subjects were exposed to different ambient levels of humidity and Schirmer tests were performed with and without an encasing sheath, designed to reduce evaporation from the strip during the test. It was expected that the lower levels of relative humidity (RH) would result in an increased loss of wetting length from the Schirmer strip. The results showed that the wetting length was greater on the sheathed side in 31/32 tests. This difference between the wetting lengths in the sheathed and unsheathed Schirmer test strips were found to be significant at each level of RH tested. However, disappointingly, no significant difference was found in the mean difference in wetting lengths between the two eyes, compared across the four different conditions of humidity.

Various factors could have contributed to this unexpected result. It may be that the variance in wetting length between the two eyes, in the small number of subjects studied, masked the effect of sheathing on wetting length. This could have been amplified by an additional impact of repeated Schirmer tests carried out in succession. Also, for the unsheathed Schirmer test there may have been variations from test to test in the evaporation from the exposed strip, dependent on the disposition of the Schirmer paper when in position. A greater rate of evaporation would be expected from the upper surface exposed directly to ambient conditions than the lower surface, facing the skin and for this surface there would be variation from experiment to experiment, which would include variations in air currents. For the sheathed test some evaporation was likely from the region of the Schirmer strip not covered by the sheathing material, at the bend of the paper near the site of insertion. This will have varied from subject to subject and from test to test. Additionally the plastic sheathing material was assumed to be completely impermeable to water but its properties were not measured. Nonetheless, the general concept that sheathing will limit variation in the Schirmer response dependent on differences in ambient humidity and airflow is supported by the *in vitro* studies of Buckmaster and Pearce (Buckmaster and Pearce, 2016). In this experiment a method was established that involved immersing the first 5mm of a standard Schirmer strip in an unlimited water supply. Results showed a significant decrease in wetting length as the RH reduced in the unsheathed Schirmer test, ranging from 27mm at 5% RH to 39mm at 95% RH. Once the Schirmer strip was modified with a “plastic film” the wetting length did not change significantly at any of the RHs tested. More recently Radke et al., (2017) also conducted *in vitro* work in which they created a sheath for the Schirmer strip and found reduced evaporation from the strip in this condition. The group concluded that that a sheathed version of the Schirmer test should be used to obtain the best clinical results (Radke et al., 2017).

Improvements to the protocol used in the current studies would include study of a larger population and probably, use of a smaller number of RH settings on separate visits, at, say, 5%, 45% and 95% RH. The laboratory studies cited and the clinical studies reported

here suggest that sheathing of the Schirmer strip would provide a test of tear production that is more precise than that of the unsheathed test. The beneficial effect of sheathing should be equally applicable to the reflex Schirmer (Ia) and the anaesthetic Schirmer tests (Ib and II).

In the longer term, the approach must be tested in the field, using a sophisticated (standardised manufacture with defined properties of the material to evaporation) version of the test in large populations, comparing variation of wetting lengths with sheathed and unsheathed versions of the test, over a wide age span. In this way it is hoped that better standardisation of the sheathed Schirmer test could be achieved, applicable in clinics independent of ambient conditions, where environments can normally be dependent on season, geography or the physical conditions of a clinic. Improvements to the technique can be envisaged, such as taking serial measurements of wetting during the test in order to derive a true flow rate (Holly, Lamberts and Esquivel, 1982; Radke et al., 2017).

The second set of experiments (Chapter 5) explored the possibility of driving down tOsm to a basal level by suppressing tear evaporation. It was predicted that such a value would be the lowest achievable in an individual and for this reason it is referred to as the basal tear osmolarity (BTO). It was further predicted that this value would lie close to and be determined by plasma osmolality (pOsm). For both reasons it was expected that the BTO would show less variance than tOsm measured in open eye conditions where osmolarity is modified by exposure to the external environment. The BTO is a measure of tOsm obtained after a sufficient period of evaporative suppression and allowing equilibration of the tears with the vascular circulation across the conjunctival epithelium.

Two scenarios of evaporative suppression were employed; eye closure and exposure to 70% RH. The results demonstrated that tOsm was reduced after eye closure and to a lesser and less significant degree in the 70% RH, in both the normal and DED groups when compared to tOsm measured in uncontrolled clinic conditions. The tOsm values obtained after evaporative suppression fell within the range accepted for normal pOsm of 285-295 mOsm/Kg, (Matz, 1996; Stookey, 2005; Cheuvront et al., 2010b). It is proposed that calculating the BTO could provide a more personal baseline against which to gauge the severity of tear hyperosmolarity in DED patients and also permit a more accurate assessment of whole body hydration of potential value in diagnosing dehydration in the elderly.

Some caution must be exercised in interpreting the results of the closed eye test in the current study here. The studies reported here should be repeated in larger groups of normal and DED subjects showing a wide range of tOsms, in order to establish the repeatability of the measure and to strengthen the conclusions that BTO can be achieved

in severe DED patients and that inter-eye differences are reduced in all subjects following exposure to evaporative suppression.

Also, the TearLab® device used to measure tOsm in these studies calculates tOsm on the basis of electrical impedence, dependent on the presence of charged ions. It therefore does not respond to the presence of urea and glucose molecules in the tears and might be expected to under-read the level of plasma osmolality/osmolarity by approximately 6-7 mOsm/L in a non-diabetic, non-uraemic subject. The precise level of this discrepancy will become apparent when future studies are conducted directly comparing closed eye BTO values with pOsm values. By including the measurement of plasma composition in such studies, the precise contribution of urea and glucose to pOsm can be identified. Although, for the reasons stated, the TearLab® value for the BTO may not precisely correspond to pOsm this would not detract from the value of the measure as a test for dehydration. Here again, a study of the BTO versus pOsm in the field, in patients experiencing clinical dehydration, should allow thresholds for the BTO relevant to different levels of dehydration to be set and compared with standard measures of assessment.

Certain improvements in the proposed test may be envisaged in the future. Collaborative studies at another site are planned, which will allow tOsm to be tracked in open-eye conditions during evaporative suppression at 95% RH. This should permit the optimum period for a closed-eye BTO test to be defined more clearly and allow the time course of tOsm restoration to be explored, on return to clinic conditions, which is of interest in DED patients. At present it is envisaged that a BTO test could aid in the detection of dehydration in the elderly in the care home environment. However, if it transpires, from future studies, that the BTO can be acquired after a short period of eye closure, say 15 minutes or less, regardless of the starting level of tOsm, then the utility of the test will be greatly enhanced and it could find a place in the study of body hydration in sports medicine, under laboratory conditions (Ungaro et al., 2015) and in field conditions, in both sports medicine (Holland et al., 2017) and military environments (Sollanek et al., 2012).

The third set of experiments (Chapter 6) addressed the relative contribution of each eye to the reflex secretory response using the Schirmer test in normal subjects. There are many instances that can lead to unilateral trigeminal sensory loss that can be temporary or permanent, for example LASIK, trigeminal nerve section or ablation, *Herpes zoster ophthalmicus* or *Herpes simplex* keratitis. It is currently unknown as to what extent the sensory drive from a normal eye can compensate for any defective lacrimal response in the fellow affected eye, and hence compensate and limit the risk of DED developing. It was hypothesised that in the steady state, when the eyes were open and exposed to the environment, sensory inputs from each eye stimulate lacrimal secretion both ipsilaterally and contralaterally, via central connections of the trigeminal nerve with the superior

salivatory nucleus (SSN) on each side. This arrangement was referred to as sensory cross-innervation. The results presented here, as in other reports, showed that bilateral topical anaesthesia resulted in a bilateral reduction in Schirmer wetting length (Lamberts, Foster and Perry, 1979; Jordan and Baum, 1980). Following unilateral topical anaesthesia, the effects on the Schirmer wetting length were in the direction predicted, i.e. assuming a sensory cross-innervation to the SSN. There was a fall in secretion in the anaesthetised eye that was less than that which occurred when there was bilateral anaesthesia and the wetting length in the fellow, unanaesthetised, eye was lower than in its control eye following bilateral saline. However, the differences shown in each case were not significant. Despite the lack of significance the concept of sensory cross-connectivity cannot be totally ruled out, but the current study suggests that if there is an input from the contralateral ocular surface, it is limited compared to that from the contralateral nasal mucosa, as discussed in the papers by Heigle and Pflugfelder, (1996) and Gupta et al., (1997) (Gupta, Heigle and Pflugfelder, 1997; Heigle and Pflugfelder, 1996). As mentioned in Chapter 7, this could have reflected a problem created by instillation of two drops prior to conducting the Schirmer test. If the instilled drops were not cleared adequately prior to the test, the wetting length could have been extended artefactually. Another factor affecting the lack of significance may have been the small subject numbers.

The difficulty related to drop instillation could be circumvented if subjects with dense, unilateral trigeminal anaesthesia as present in neurotrophic keratitis (NK), were recruited and exposed to the desiccating environment of 5% RH. Following the induction of topical anaesthesia in the normal, fellow eye, it would be possible to follow the response of the NK eye to exposure without the complication of instilling a drop into the clinically anaesthetic, NK eye. This would remove the problem of the original protocol in this experiment that anaesthetic drop instillation might dilute the tears and mask the effect of desiccation on tOsm. The use of fluorophotometry instead of the Schirmer test would also remove the reflex tearing effect inherent in the latter approach that has the potential to confound the results. A further expansion of this study could also investigate how combinations of nasal mucosal and ocular anaesthesia influence the steady state level of lacrimal secretion. The purpose of that study would be to further explore how the sensory inputs from each eye are integrated centrally to influence the ipsilateral and contralateral steady state tear production and to what degree the nasal mucosa contributes.

The fourth experiments (Chapter 7) monitored tOsm under conditions of desiccating stress, with and without anaesthesia. The use of topical anaesthetics was designed to create a sensory blockade of signals from the cornea, allowing the effects of defined levels of stress on tOsm without secretory compensation via the LFU to be determined.

This approach was designed to reinforce understanding of the mechanism of DED. A study by Abusharha and Pearce (2013) suggested that in normal subjects, compensatory mechanisms, stimulating lacrimal secretions via the LFU, were able to prevent a significant rise in tOsm following exposure to low RH (Abusharha and Pearce, 2013). These responses in subjects with normal eyes would be a guide to the expectations of such individuals to cope with desiccating stress encountered in everyday life. In this experiment it was anticipated that in the DED patients a rise in tOsm would occur, superimposed on the already raised value that characterises DED, as a result of the increased evaporation induced by the desiccating stress. The effect was expected to be amplified further in these subjects after sensory blockade where sufficient reserves of LFU compensation would have already been utilised. Unexpectedly, the results did not show any significant increase in tOsm at the height of the anaesthetic effect (approximately 30 minutes) in either the normal or DED groups. It is possible that integrity of the pathway linking the nasal mucosa to the lacrimal secretory system was involved in this paradoxical response. A number of recent studies have confirmed, in both animals (Kossler et al., 2015; Brinton et al., 2015) and humans (Friedman et al., 2016; Gumus and Pflugfelder, 2017) that controlled nasal mucosal stimulation, of a mild degree, stimulates tear secretion. It is possible that in the experiments described, stimulation of the nasal mucosa by the desiccating condition of 5% RH compensated for the decrease in tear production that would otherwise have occurred due to withdrawal of sensory drive from the ocular surface. It would therefore be of interest to repeat the experiments during a period of combined, bilateral, nasal and ocular anaesthesia.

Despite a lack of change to the tOsm after exposure to desiccation, temporary punctate epithelial keratitis (PEK) was recorded in the Sjögren syndrome subjects following instillation of anaesthetics. This suggested that the tOsm values sampled from the tear meniscus were not necessarily representative of the tOsm occurring over the whole of the central cornea. Previous publications have reported that tOsm is not evenly distributed between the tear compartments of the conjunctival sac, preocular tear film and tear meniscus (Bron, Yokoi and Gouveia, 2002; Gaffney et al., 2010). With rapid evaporation and thinning of the exposed preocular tear film, it has been purported in models that tOsm could reach up to 900 mOsm/L (King-Smith et al., 2008). This could contribute significantly to localised interpalpebral increases in tOsm especially between blinks (Liu et al., 2009) and create conditions sufficient to induce epithelial cell damage. Further investigation into the variation in tOsm in the different compartmental areas following episodes of desiccating stress is warranted, although instrumentation presently available for measuring tOsm may limit this possibility. There is currently no method to measure tOsm in the precorneal tear film or in the corneal epithelium itself. A device for the measurement of conjunctival osmolarity is available, but its accuracy is yet to be

established (Rocha et al., 2017). In future research that expanded on this study and incorporated a larger dataset, further analytical methods would be appropriate to use, for example a mixed ANOVA or two-way ANOVA. In addition, further information about demographics and other covariates could be collected. With this data a linear mixed model with random intercepts could be applied to explain any changes in the tOsm measures over time.

The body of work presented here derives from a set of pilot studies, with small numbers of subjects in each experiment. It is acknowledged, therefore, that interpretations and conclusions drawn must be provisional and that the novel concepts presented will require further validation. The means to achieve this and some areas for future research have been outlined above.

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APPENDIX I

Anglia Ruskin University
Cambridge & Chelmsford

INFORMATION SHEET

Postgraduate Medical Institute

A Pilot Study in Tear Dynamics: Tear Osmolarity and Reflex Features of the Lacrimal Functional Unit

veru visionandeyeresearchunit

Introduction

We are inviting you to take part in a research project relating to dry eyes. This research is being carried out as part of a PhD project. In order to help you decide whether to volunteer, these notes describe the research and what it would involve for you if you took part. Please read them carefully and let us know if there is anything that is unclear.

Dry Eye

Dry Eye (DE) is a very common eye condition affecting, 5-35% of the population worldwide. It may give rise to symptoms, such as burning and grittiness, light sensitivity and blurred vision. Symptoms can be worsened by dry environments such as those produced by air-conditioning or by computer use, which slows the blink rate. Symptoms are more prevalent in women and DE is more common with increasing age.

Dry eye is caused by evaporation of water from the tear film, due either to a failure of tear secretion or to a loss of the normal barrier to evaporation. The increased salt concentration of the tears that results, (hyperosmolarity), stimulates inflammation at the surface of the eye which is a part of the disease. To some extent a reflex system that regulates tear production can compensate for drying, but when this fails, damage to the surface of the eye results.

What is the purpose of the project?

The project is designed to show how the eye regulates tear production in a protective way, when exposed to drying environments. To do this we will take measurements in carefully controlled conditions of low humidity or increased air flow, in people with normal eyes and in patients with dry eye disease and other disorders affecting the surface of the eye.

What will happen to the results of the research project?

The results of the research will be presented in a thesis and will be the basis of research publications. Data from the study may also be used as the basis of further research. Anonymised data will be shared with TearLab, a company supporting the research. TearLab will provide equipment essential to the study but are not involved in the collection of data.

INFORMATION SHEET

Postgraduate Medical Institute

A Pilot Study in Tear Dynamics: Tear Osmolarity and Reflex Features of the Lacrimal Functional Unit

Why have I been chosen?

You have been asked to join this study because your eyes satisfy the inclusion criteria of the study, either as a subject with normal eyes, or someone with a disorder that affects the surface of the eyes, such as DE.

Do I have to take part?

Involvement in the study is voluntary. You may discontinue at any point without giving a reason for withdrawal. If you decide to withdraw, then any data that we have collected can be removed from use in the project up to two weeks after it has been collected.

What are the possible benefits of taking part?

There are no direct benefits to you as a participant, but it is expected that the study will provide us with new information about the mechanism of dry eye disease. This will allow us to develop new approaches to therapy. There will therefore be a benefit to the wider community.

What are the possible disadvantages and risks of taking part?

The study requires you to attend for several visits and to have various procedures performed while you sit in our environmental chamber for up to an hour at a time. Details of the procedures are provided later in this information sheet. There may be brief discomfort when different eye drops are used but while some tests can on occasion be uncomfortable they are not painful.

What if there is a problem?

We do not foresee any risks associated with your participation in the study and all measurements will be carried out by a qualified member of the research team. Some transient changes may be observed at the surface of the eye at the end of a session. These are reversible and if they occur you will be provided with eye drops to use, for a limited period of time and your GP will be advised.

What do I have to do?/ what will happen to me if I take part?

You will be required to take part in four, three hour sessions, these sessions will include a suitable break for a meal and relaxation. Your travel expenses up to £10 per visit can be provided. During the sessions you will first be required to adapt in the environmental chamber for 10 minutes, usually with your eyes closed and the eyes will then remain open for the rest of the session. You may also be in the chamber with 1 other participant, plus the researchers. The environmental chamber is a well-lit room about 3 metres long and 2 metres wide in which we are able to very accurately control the temperature and humidity, it also makes a constant humming sound.

We will record how the surface of your eyes responds to a) different humidities, airflows and temperatures b) and how your response is affected by the use of anaesthetic eye drops. To do this, we will from time to time take photographs of the eyes, sample the tears or instil eye drops to aid us in assessing the response of your eyes. You will also be asked about the comfort of your eyes.

The following list includes tasks that you might be required to perform as part of the study. You will be asked to sit in the chamber for up to 3 hours-a constant humming sound will be present in the background. While you are in the chamber, we may:

1) Ask you to fill out a questionnaire about the comfort of your eyes.

2) Sit you at a slit-lamp microscope which will allow us to examine and take photographs of the eyes with magnification.

3) Shine bright lights into the eyes to assist our assessments; instil eye drops and some non permanent dye, fluorescein. Some drops may sting slightly.

4) Have a sample of tears taken from the lower eyelid. This is not uncomfortable.

5) Measure your tear production with the Schirmer test, which involves the insertion of a small strip of filter paper under the lids on each side to collect the tears. This takes 5 minutes and may be uncomfortable but not painful.

Do I need to prepare for the study?

If you are a contact lens wearer we ask that you do not wear your lenses for at least 8 hours before participating in the study. Cosmetics must not be used on the day of assessment and if you are a dry eye patient you must not use any artificial tears for at least 2 hours before your visit. If you are taking any medication for a systemic condition and the doses are likely to remain stable for the duration of the study, there is no action to take.

Will my taking part in this project be kept confidential?

All information collected in this study will be anonymous and you will not be identified in any reports or publications. To achieve this your details will be coded and only the research team will have access to your documents and data (Catherine Willshire and Professors Bron and Buckley).

What if anything goes wrong/how to make a complaint?

This study will be carried out by a qualified Optometrist who will be at hand to resolve any problems that occur as part of the data collection. We do not anticipate any such circumstances as all the measurements form part of standard eye examination.
If you would like to make a complaint, this can be made through the project supervisors (Professors Bron and Buckley) or via the University Secretary and Clerk:

All complaints will be listened to sympathetically.

Who has reviewed this study?

NHS Ethics Committee: South East Coast-Brighton and Sussex.

Who is organising/ funding the research?

The research is organised and funded by Anglia Ruskin University.

Any further queries?

If you need any further information, please contact Catherine Willshire e-mail:

If you decide to take part you will be given this information sheet and asked to sign a consent form.

APPENDIX II

CONSENT FORM

Postgraduate Medical Institute

A Pilot study of Tear Film Dynamics: Tear Osmolarity and Reflex Features of the Lacrimal Functional Unit

Name		Code	
IRAS No.		Centre	ARU
GP		Surgery	
Main Researcher: Catherine Willshire		e-mail:	

I. I confirm that I have read the information sheet dated…… (version……) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

II. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected (See Page 2). ☐

III. I understand that relevant sections of my medical notes and data collected during the study may be looked at by members of the research team. I give permission for these individuals to have access to my records. ☐

IV. I understand that the information collected about me will be used to support other research in the future, and may be shared anonymously with TearLab (a third party supporting the research) ☐

V. I agree to my General Practitioner being informed of my participation in the study ☐

VI. I understand that the confidentiality of the information I provide will be safeguarded and all data will be anonymised or coded to comply with the Data Protection Act. ☐

VII. I understand that I can withdraw permission to use the data within two weeks of the initial measurements, in which case the material will be deleted (See Page 2). ☐

VIII. I agree to take part in the above study. ☐

IX. I would like to receive a summary of the study once the project has been completed (if so, please provide a postal address below) ☐

……

Name of Participant	Signature	Date
Name of Person taking consent	Signature	Date

I you wish to withdraw your participation in the study or the permission to use your data within the research, please compete the form below and return to: Catherine Willshire, VERU, Eastings, Anglia Ruskin University, East Road, Cambridge, CB1 1PT.

Please tick the appropriate statement:

a) I wish to withdraw my participation from the study named above ☐

Name of Participant	Signature	Date

b) I wish to withdraw permission to use the data collected in the study named above (this must be received a maximum of two weeks after the collection of the initial measurements) ☐

Name of Participant	Signature	Date

APPENDIX III

Anglia Ruskin University

Postgraduate Medical Institute

veru

Dear General Practitioner,

This letter is to inform you that a patient registered at your practice has volunteered to take part in a research project entitled 'A Pilot Study of Tear Dynamics: Tear Osmolarity and Reflex Features of the Lacrimal Functional Unit', to be conducted at Anglia Ruskin University, Cambridge in the Vision and Eye Research Unit.

The project is aimed at gaining a better understanding of how the eye reacts to drying environments, caused by reduced humidity or increased airflow at a given temperature. We are interested in assessing both normal subjects without eye disease and those with Dry Eye.

Your patient has been approached because they satisfy the inclusion criteria for the project.

We do not foresee any risks associated with participation in the study and all measurements will be carried out by a qualified member of the research team. Most of these form the basis of a standard assessment for dry eye. However, if any adverse events occur the patient will be provided the appropriate treatment at the time and you will be informed.

Please contact me if you require any further information.

Yours sincerely,

Ms C Willshire BSc (Hons) MCOptom

APPENDIX IV

Ocular Surface Disease Index© (OSDI©)[2]

Ask your patients the following 12 questions, and circle the number in the box that best represents each answer. Then, fill in boxes A, B, C, D, and E according to the instructions beside each.

Have you experienced any of the following *during the last week*?	All of the time	Most of the time	Half of the time	Some of the time	None of the time
1. Eyes that are sensitive to light? . .	4	3	2	1	0
2. Eyes that feel gritty?	4	3	2	1	0
3. Painful or sore eyes?	4	3	2	1	0
4. Blurred vision?	4	3	2	1	0
5. Poor vision?	4	3	2	1	0

Subtotal score for answers 1 to 5 (A)

Have problems with your eyes limited you in performing any of the following *during the last week*?	All of the time	Most of the time	Half of the time	Some of the time	None of the time	N/A
6. Reading?	4	3	2	1	0	N/A
7. Driving at night?	4	3	2	1	0	N/A
8. Working with a computer or bank machine (ATM)?	4	3	2	1	0	N/A
9. Watching TV?	4	3	2	1	0	N/A

Subtotal score for answers 6 to 9 (B)

Have your eyes felt uncomfortable in any of the following situations *during the last week*?	All of the time	Most of the time	Half of the time	Some of the time	None of the time	N/A
10. Windy conditions?	4	3	2	1	0	N/A
11. Places or areas with low humidity (very dry)?	4	3	2	1	0	N/A
12. Areas that are air conditioned? . . .	4	3	2	1	0	N/A

Subtotal score for answers 10 to 12 (C)

Add subtotals A, B, and C to obtain D (D = sum of scores for all questions answered) (D)

Total number of questions answered (do not include questions answered N/A) (E)

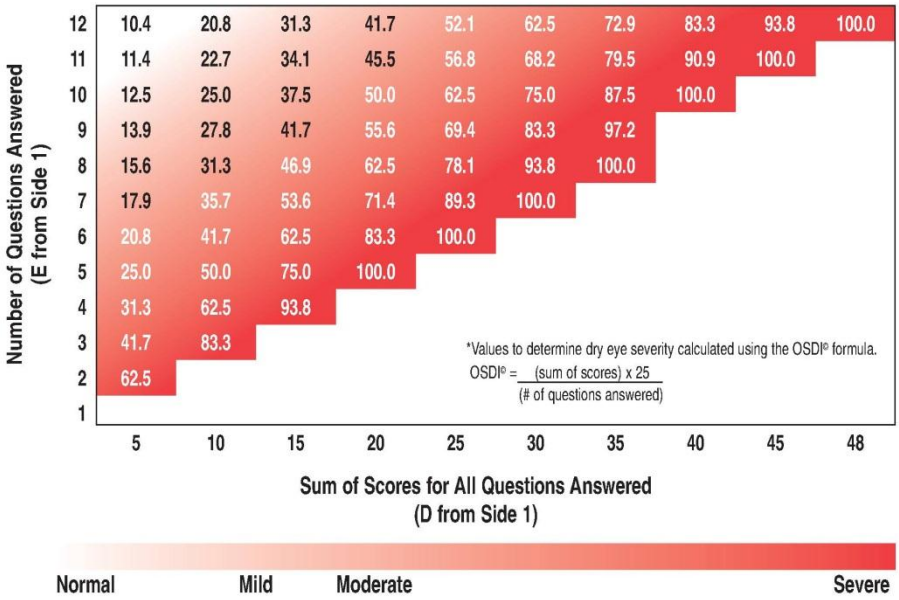
Please turn over the questionnaire to calculate the patient's final OSDI© score.

Evaluating the OSDI® Score[1]

The OSDI® is assessed on a scale of 0 to 100, with higher scores representing greater disability. The index demonstrates sensitivity and specificity in distinguishing between normal subjects and patients with dry eye disease. The OSDI® is a valid and reliable instrument for measuring dry eye disease (normal, mild to moderate, and severe) and effect on vision-related function.

Assessing Your Patient's Dry Eye Disease[1, 2]

Use your answers D and E from side 1 to compare the sum of scores for all questions answered (D) and the number of questions answered (E) with the chart below.* Find where your patient's score would fall. Match the corresponding shade of red to the key below to determine whether your patient's score indicates normal, mild, moderate, or severe dry eye disease.



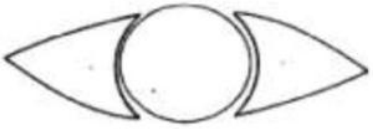
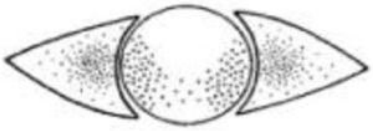
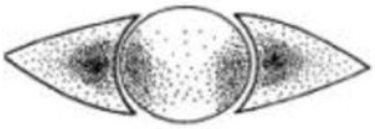
Patient's Name: _____ Date: _____

How long has the patient experienced dry eye disease? _____

Eye Care Professional's Comments: _____

1. Data on file, Allergan, Inc.
2. Schiffman RM, Christianson MD, Jacobsen G, Hirsch JD, Reis BL. Reliability and validity of the Ocular Surface Disease Index. *Arch Ophthalmol.* 2000;118:615-621

APPENDIX V

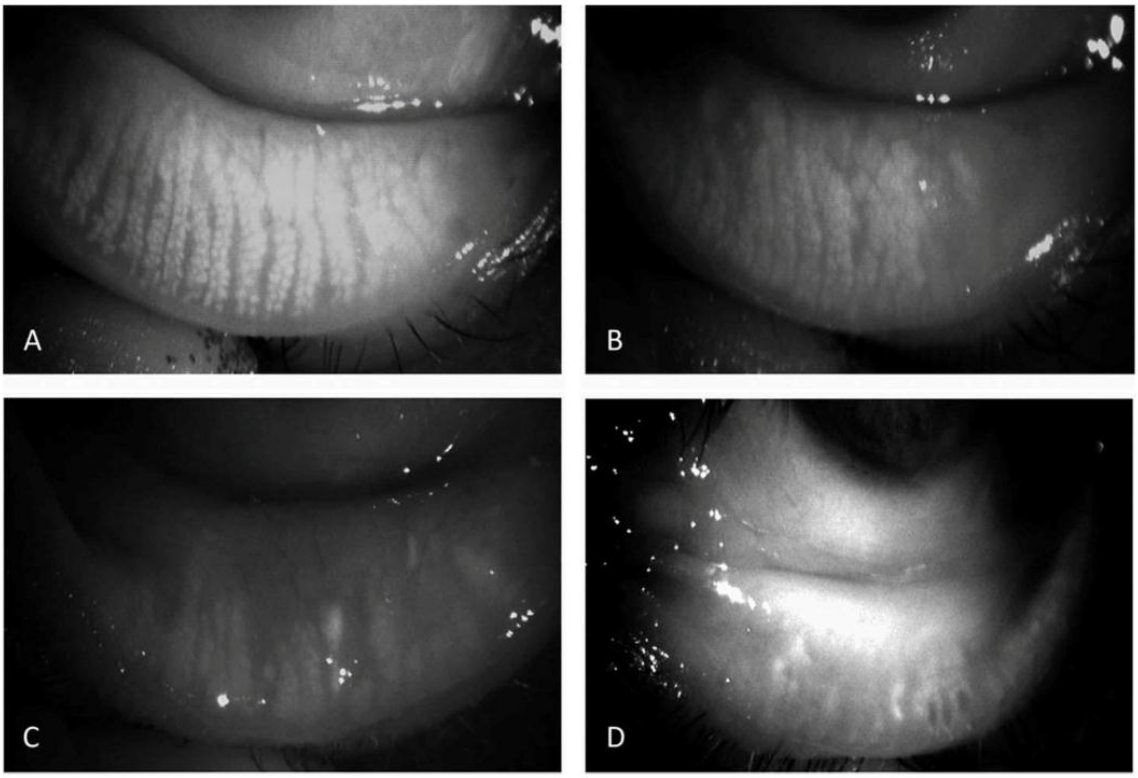
Staining appearnace	Grade	Verbal descriptor
	0	Absent
	I	Minimal
	II	Mild
	III	Moderate
	IV	Marked
> IV	V	Severe

APPENDIX VI

Meiboscore

The lost areas of MGs were scored from 0 to 3.

Grade 0	Represents no loss of MGs (white area) (A)
Grade 1	Represents a lost area (black area) less than one third of the total area (B)
Grade 2	Represents a lost area between one-third and two-thirds of the total area (C)
Grade 3	Represents a lost area more than two-thirds of the total area (D)



APPENDIX VII

MONDAY 11am

RECRUITMENT RECORD CARD

Date: 25/1/16

Patient ID

Mr/Ms/Mrs/Miss
First Name:
DOB:
E-mail:
GP name and address:

Male/Female
Surname:
Age:
Contact number:

History and Symptoms

UCVA **Bin** 6/18 -1 **BCVA** **R** 6/6 -1 **L** 6/6

Do you have any history of the following eye conditions?

Condition	Yes	No	Right	Left	Further details e.g. duration and timescale
Sjögrens syndrome	(Y)	N	OD	OS	Diagnosed 2014
Dry eye	(Y)	N	OD	OS	"
Other conditions/treatments: glasses wearer from 40yrs old.					

Do you currently wear any contact lenses? **Y / (N)**

If yes please provide details of what type and how often you wear the lenses:

Wear Time	Last worn		Type/Brand	Solutions	Prescription
/7 days per week		R			
/24 hours per day		L			

Do you suffer with any of the following systemic conditions?:

Condition	Yes	No	Details e.g. duration and timescale
Hayfever	Y	N	

Any other health problems:

- Raynaud's syndrome
- Hypothyroidism

Are you taking any medication?:

If yes please provide the names of the medication, details of how long you have taken the medication and how long you have been instructed to take the medication.

- Thyroxine
- Adalat
- Lanzaprazole
- Lumecane

used at least once a day.

Any of the following?	
Oral Contraceptive	Anti-biotics
Anti-histamine	Decongestants
HRT	Anti-inflammatories
Anti-depressants	B-blockers/anti-hypertensive
Diuretics	Tranquilizers
Anti-convulsants	Statins/Aspirin

Length of treatment: Continuous

Duration of treatment throughout study: Y / N

Diagnosis:

DE- ADDE ☐ EDE ☐ Sjögrens ☑ Normal ☐

Suitable for study:

Yes ☑ No ☐

If Yes, number of study Project 1 or 2.

If No, please state reason

If Yes, please give participant an information sheet and assign a random code

Code ☐

Baseline measurements

Clinic conditions

Temperature 24°c Relative Humidity 58%

Refraction and VA

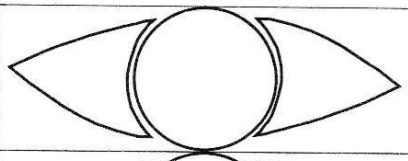
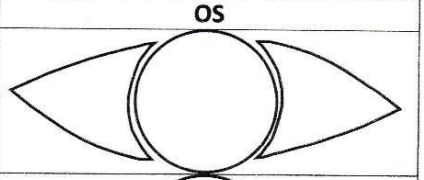
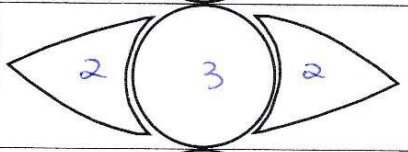
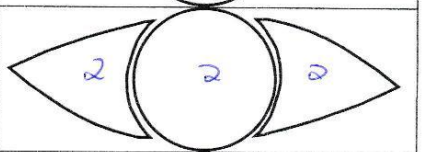
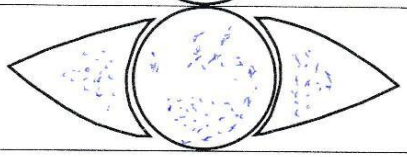
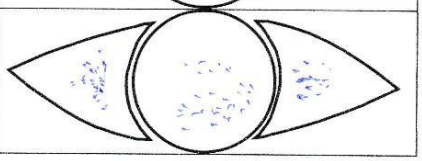
	UCVA	Sph	Cyl	Axis	Add	Δ	BCVA
R	6/18	+3.25	-0.75	110	+2.25		6/6 -1
L		+3.00	-0.75	70	+2.25		6/6

c glasses - varis (approx. 1yr old)

OSDI Questionnaire Score 43.75

	R	L
Osmolarity	318	331
TBUT	3.69 2.84 3.31 | Av. 3.28	2.66 2.60 2.25 | Av. 2.50
Schirmer (attach)	 2mm.	 2mm

Anterior Ocular Health

	OD	OS
White light		
New Oxford grading scale	 2 3 2	 2 2 2
Fluorescein staining		

Meibomian Glands: Quality of expression Gr. 0 (Clear)
Expressibility Gr. 2 (Moderate)

APPENDIX VIII

INPUT

Project 4-record card

Subject ID
Code_____

Date 9/12/15
Diagnosis N

The effect of desiccation on the wetting length of the Schirmer test in open eye conditions and the effect of shielding the paper from evaporation

CEC
Target Temperature: 23°C
Target Relative Humidity: 45%
Time entering the CEC 1.40pm.
Time exiting the CEC 2.20pm

Examiners present C.W

clinic conditions 27°C/30% RH.

osmo: R 307 · L 303

EYES OPEN FOR 10 MINUTES-spontaneous blinking

The eye receiving the standard Schirmer strip will be randomly selected.

		Time	R UNSHIGATHED	L SHIGATHED
Schirmer	Zero		*Insert Schirmer strip in lower fornix R then L-eyes closed*	
	5 mins		*Remove Schirmer strip after 5 mins, measure and attach below*	
			 7mm	 15mm

APPENDIX IX

8/8/16

Project 1-record card (visit 1)

Subject ID

Code_____

Date 20/7/16

Diagnosis N

i. **To compare nascent lacrimal fluid osmolarity in normal subjects and DE patients, with tear osmolarity measured in clinic and standard room conditions.**

Clinic conditions

Temperature 23.3°C Relative Humidity 62%

Osmolarity R 297 L 299

CEC

Target Temperature: 23°C Examiners present C.W.

Target Relative Humidity: **45%**

Time entering the CEC 9.20am. Time exiting the CEC 10.50am

EYES CLOSED FOR 45 MINUTES-eyes moving

Osmolarity immediately on eye opening:

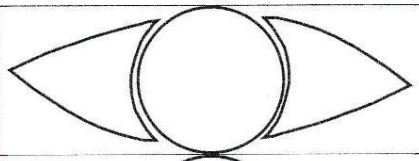
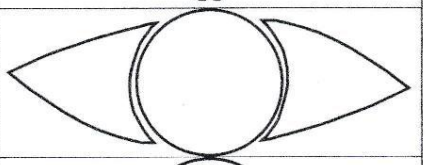
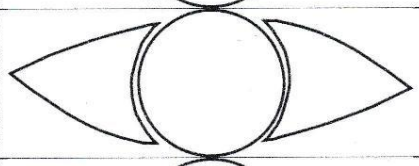
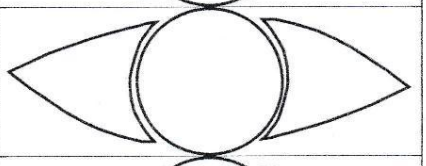
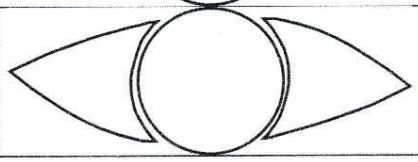
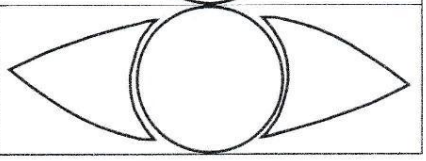
	Time	R	L
Zero		286	277

EYES OPEN for 45 MINUTES- spontaneous blinking

Osmolarity:

	Time	R	L
15 mins		297	287
30 mins		291	299
45 mins		292	292

Ocular surface health: New Oxford Grading scale

	OD	OS
White light dotting and		
Fluorescein staining		
Fluorescein staining		

Project 1-record card (visit 2)

Subject ID
Code_____

Date 21/3/16
Diagnosis N

ii. To derive values for nascent lacrimal fluid osmolarity for normal and DE subjects based on repeat measurement in conditions of high humidity

Clinic conditions
Temperature 25°C Relative Humidity 28%

Osmolarity R 301 L 304

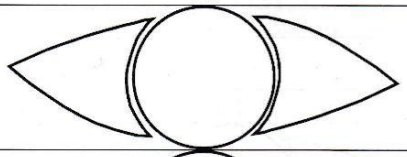
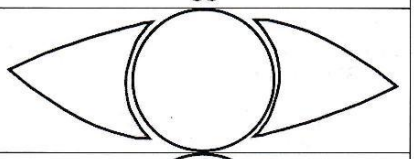
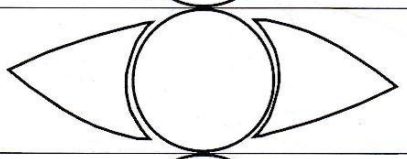
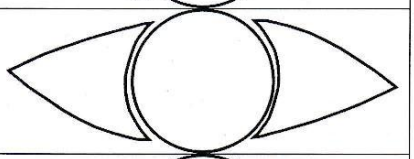
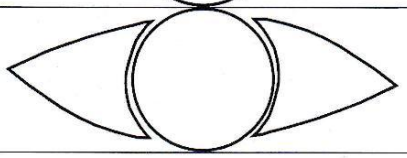
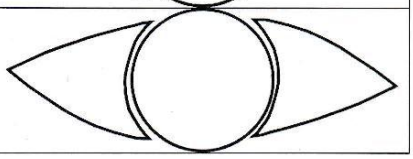
CEC
Target Temperature: 23°C Examiners present C.W
Target Relative Humidity: **70%**
Time entering the CEC 12.15pm. Time exiting the CEC 1pm

EYES OPEN-spontaneous blinking

Osmolarity:

	Time	R	L
15 mins		303	300
30 mins		299	293
45 mins		298	296

Ocular surface health: New Oxford Grading scale

	OD	OS
White light dotting and		
Fluorescein staining		
Fluorescein staining		

APPENDIX X

INPUT

Project 3-record card

Subject ID Date 9/12/15

Code_____ Diagnosis N

a. Schirmer test control: unanaesthetised eyes

CEC

Target Temperature: 23°C Examiners present C.W.

Target Relative Humidity: 45%

Time entering the CEC 11.40 am

Time exiting the CEC 12.40 pm

Clinic conditions

25°C 28%

293 289.

EYES OPEN FOR 10 MINUTES- eyes moving

		Time	R	L
Osmolarity	Eye opening			
	Zero		*Instil Saline 1 drop BE*	
	+30 secs		*Instil Saline 1 drop BE*	
Schirmer	5 mins		*Insert Schirmer strip in lower fornix R then L*	
	5 mins		*Remove Schirmer strip, measure and attach below*	
			 28mm	 29mm

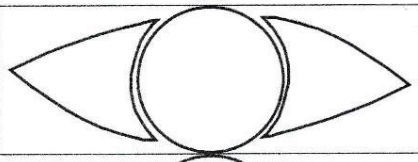
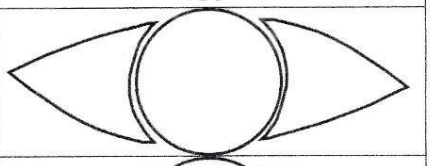
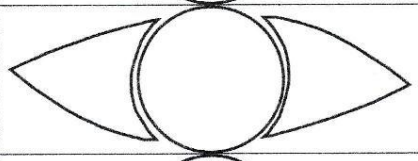
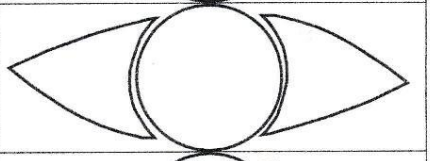
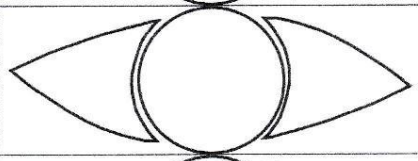
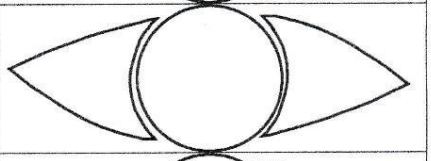
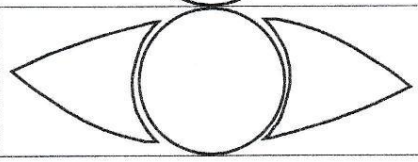
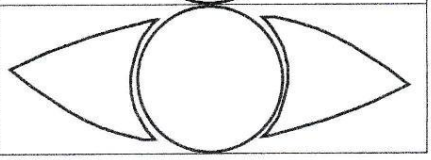
9/12/15

b. (i) Unilateral afferent block INPUT

Topical anaesthetic will be instilled into one eye and saline into the other, this will be randomly selected. (R) anaesthetic

		Time	R	L
Osmolarity	Eye opening			
	Zero		*Instil Saline 1 drop ~~R~~ (L) and 1 drop Proxy 0.5% (R) ~~L~~*	
	+30 secs		*Instil Saline 1 drop ~~R~~ (L) and 1 drop Tetra 1% (R) ~~L~~*	
Schirmer	5 mins		*Insert Schirmer strip in lower fornix R then L*	
	5 mins		*Remove Schirmer strip, measure and attach below*	
			 15mm	 34mm

Anterior Ocular Health

	OD	OS
White light		
New Oxford grading scale		
Fluorescein staining		
Lissamine Green		

✓ INPUT

CEC Date 19/12/15

Target Temperature: 23°C Examiners present C-W

Target Relative Humidity: 45%

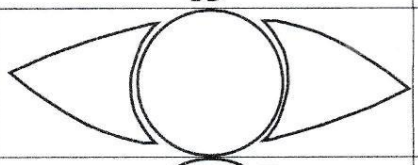
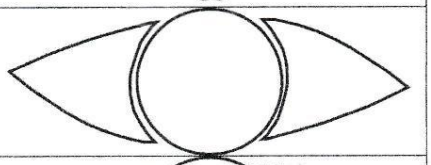
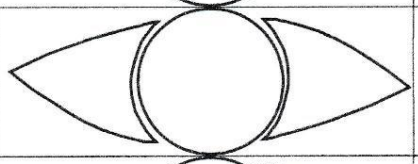
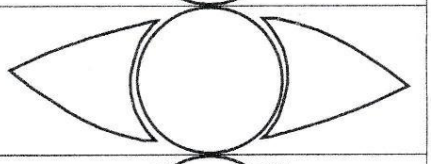
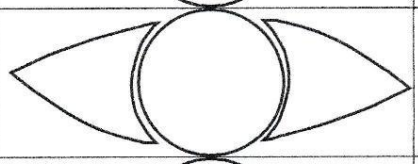
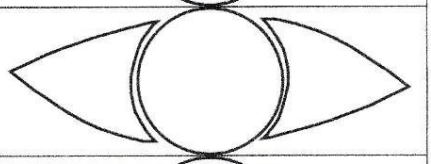
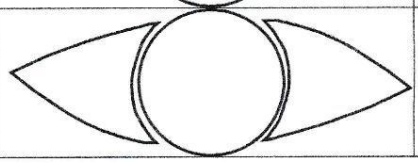
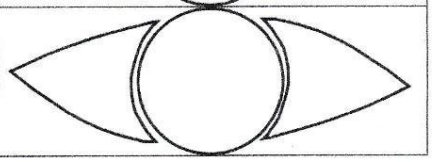
Time entering the CEC 11:30am Time exiting the CEC 12:10pm

EYES OPEN FOR 10 MINUTES-eyes moving

b. (ii) Bilateral afferent block (on subsequent day)

		Time	R	L
Osmolarity	Eye opening			
	Zero		*Instil Proxy 0.5% 1 drop BE*	
	+30 secs		*Instil 1 drop Tetra 1% BE*	
Schirmer	5 mins		*Insert Schirmer strip in lower fornix R then L*	
	5 mins		*Remove Schirmer strip, measure a... attach below*	
			 14mm	 16mm

Anterior Ocular Health

	OD	OS
White light		
New Oxford grading scale		
Fluorescein staining		
Lissamine Green staining		

APPENDIX XI

Project 2-record card

Subject ID Date 23/2/16

Code Diagnosis N

a. The effect of desiccating stress in normal subjects with an intact LFU

CEC

Target Temperature: 23°C Examiners present C.W.

Target Relative Humidity: 5%

Time entering the CEC 2.15 pm

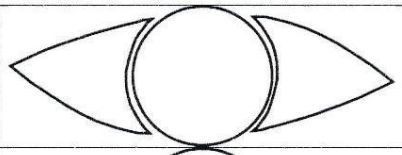
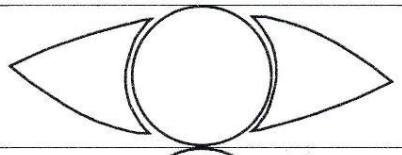
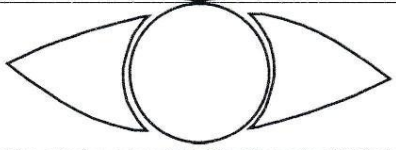
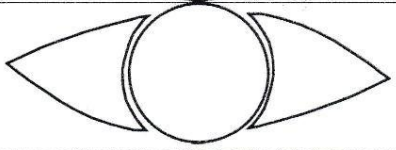
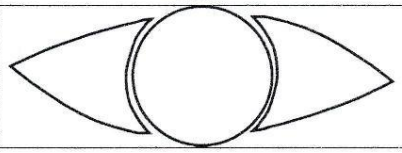
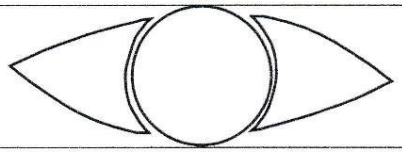
Time exiting the CEC 3.25 pm

EYES OPEN FOR 10 MINUTES-eyes moving

Osmolarity: clinic conditions 22°C / 26% RH

	Time	R	L
Clinic conditions		301	296
Zero		*Instil saline 1 drop BE*	
+30 secs		*Instil saline 1 drop BE*	
20 mins		298	293
40 mins		299	288
60 mins		288	291

Ocular surface health: Oxford Grading scale

	OD	OS
White light dotting and scoring		
Fluorescein staining dotting		
Fluorescein staining scoring		

DATE 1/31/16

b. The effect of desiccating stress on tear osmolarity in normal subjects with induced sensory blockade

CEC

Target Temperature: 23°CExaminers present C-W

Target Relative Humidity: 5%

Time entering the CEC 2:10 pm

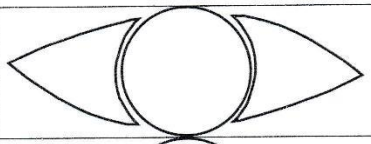
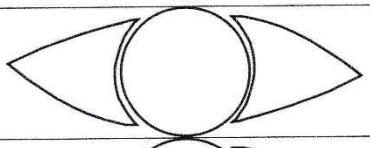
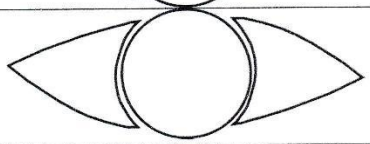
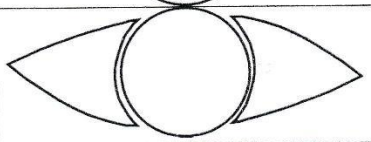
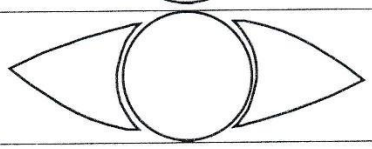
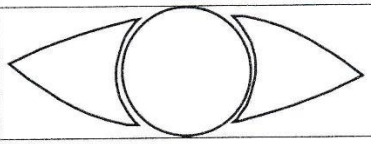
Time exiting the CEC 3:30 pm

EYES OPEN FOR 10 MINUTES-eyes moving

Osmolarity: clinic conditions 22°C / 41% RH

	Time	R	L
Clinic conditions		290	297
Zero		*Instil proxymetacaine 0.5% 1 drop BE*	
+30 secs		*Instil tetracaine 1% 1 drop BE*	
20 mins		300	301
40 mins		293	285
60 mins		288	285

Anterior ocular health:

	OD	OS
White light		
New Oxford gradingscale		
Fluorescein staining		

APPENDIX XII

Figures A12 a-d show slit-lamp photography with fluorescein (from Chapter 6) of the left corneas of the normal and DED subjects following instillation of anaesthetic and after 1 hour exposure to desiccating conditions. Figures A12 e-l show slit-lamp photography with fluorescein of the right and left corneas of the normal and DED subjects following instillation of saline and after 1 hour exposure to desiccating conditions.

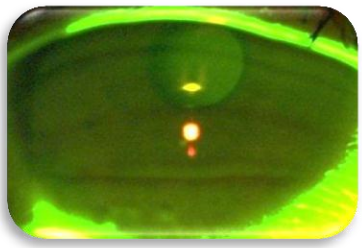
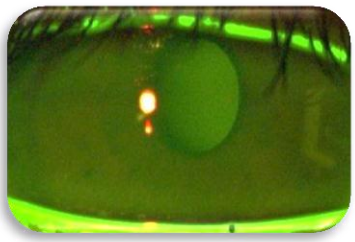
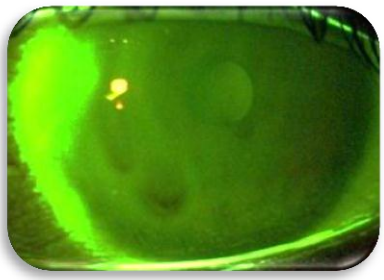
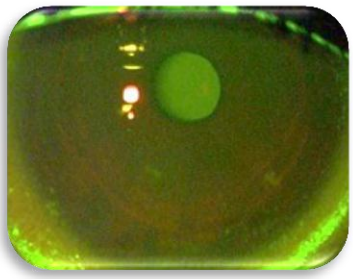
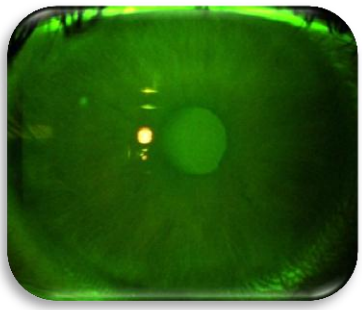
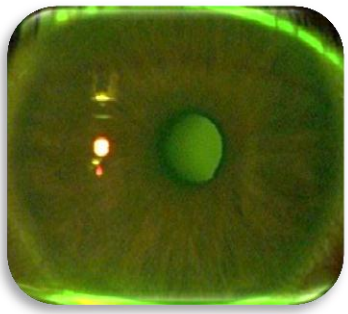
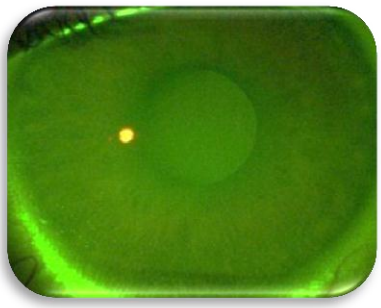
Subject 1 **Normal**	 **PRE-EXPOSURE**	 **POST-EXPOSURE**
Subject 2 **Normal**	 **PRE-EXPOSURE**	 **POST-EXPOSURE**
Subject 3 **Normal**	 **PRE-EXPOSURE**	 **POST-EXPOSURE**
Subject 4 **Normal**	 **PRE-EXPOSURE**	 **POST-EXPOSURE**

Figure A12a Slit-lamp images with fluorescein pre and post CEC exposure to desiccating conditions for 60 minutes and after anaesthetic instillation in the left eyes of normal subjects 1-4.

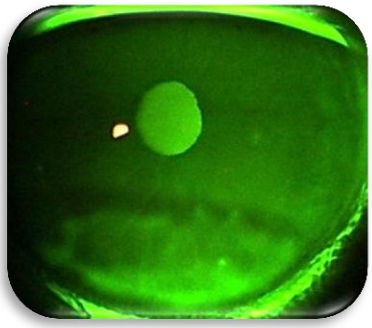
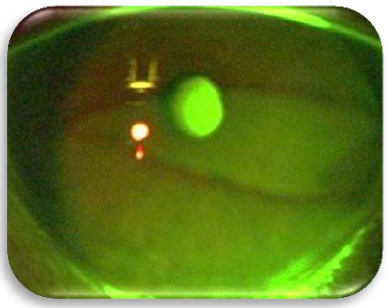
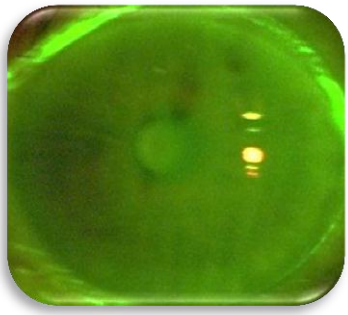
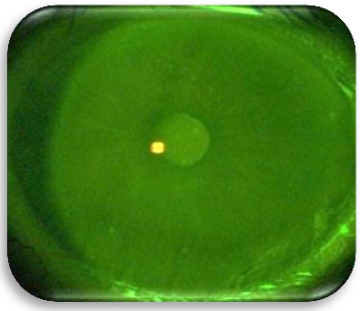
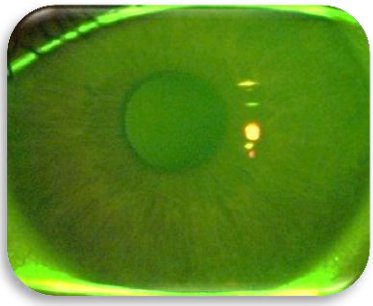
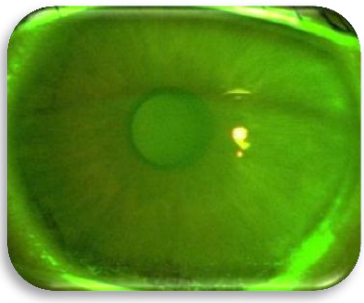
Subject 5 **Normal**	 **PRE-EXPOSURE**	 **POST-EXPOSURE**
Subject 6 **Normal**	 **PRE-EXPOSURE**	 **POST-EXPOSURE**
Subject 7 **Normal**	 **PRE-EXPOSURE**	 **POST-EXPOSURE**
Subject 8 **Normal**	 **PRE-EXPOSURE**	 **POST-EXPOSURE**

Figure A12b Slit-lamp images with fluorescein pre and post CEC exposure to desiccating conditions for 60 minutes and after anaesthetic instillation in the left eyes of normal subjects 5-8.

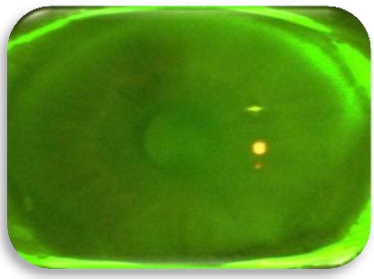
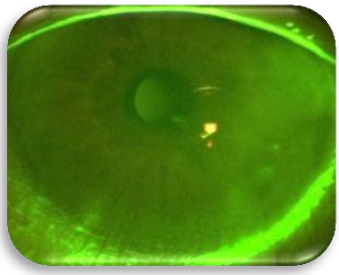
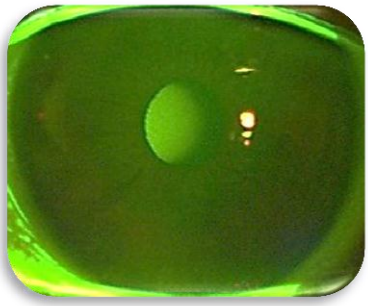
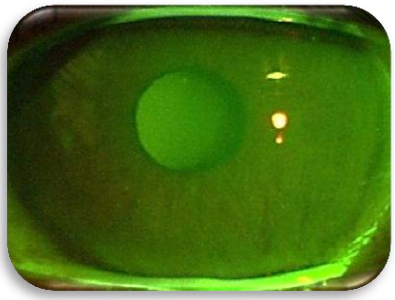
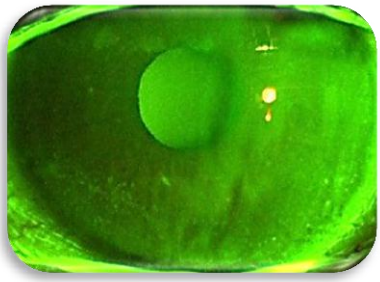
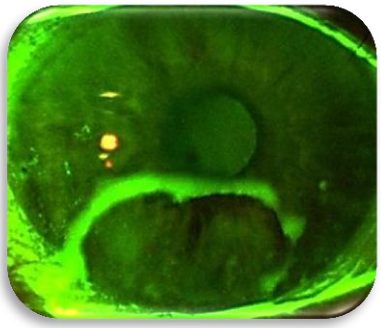
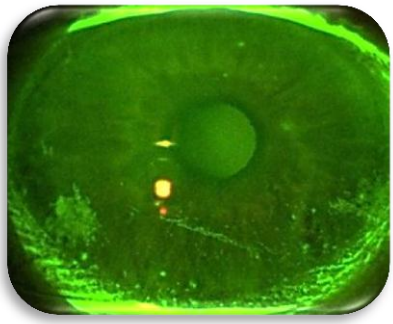
Subject 1 **DED**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 2 **DED**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 3 **DED (SjS)**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 4 **DED (SjS)**		
	PRE-EXPOSURE	**POST-EXPOSURE**

Figure A12c Slit-lamp images with fluorescein pre and post CEC exposure to desiccating conditions for 60 minutes and after anaesthetic instillation in the left eyes of DED patients 1-4.

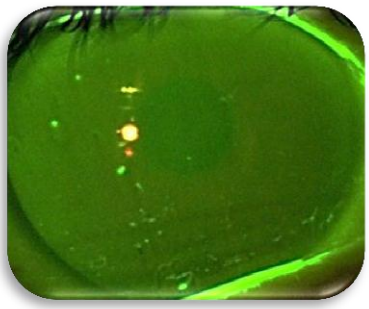
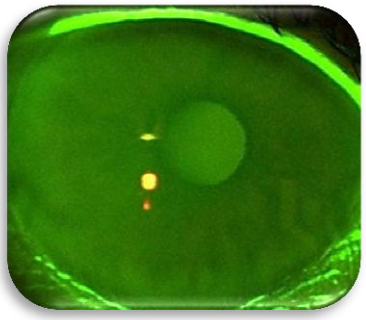
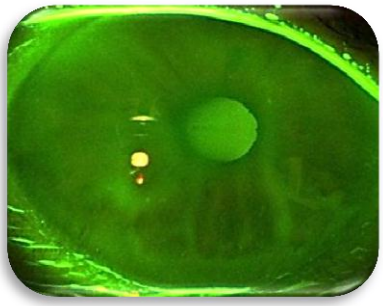
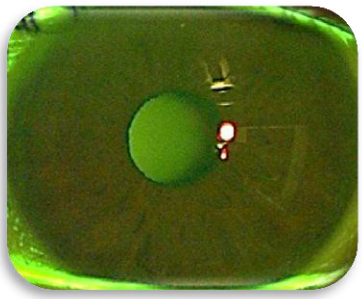
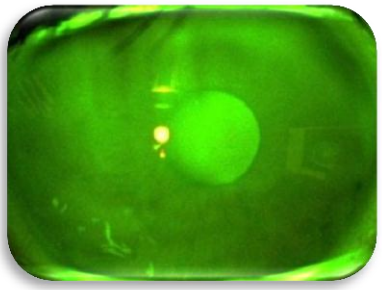
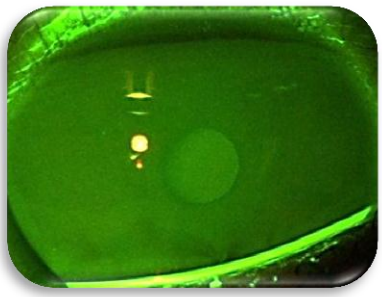
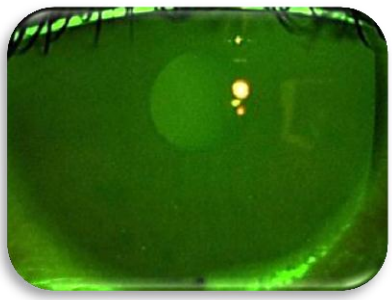
Subject 5 **DED (SjS)**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 6 **DED (SjS)**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 7 **DED**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 8 **DED**		
	PRE-EXPOSURE	**POST-EXPOSURE**

Figure A12d Slit-lamp images with fluorescein pre and post CEC exposure to desiccating conditions for 60 minutes and after anaesthetic instillation in the left eyes of DED patients 5-8.

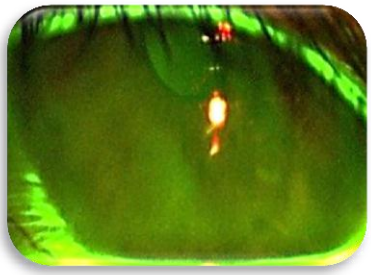
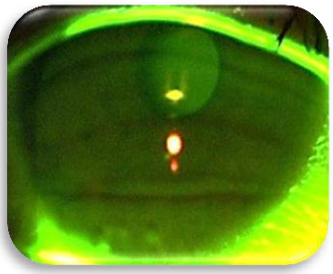
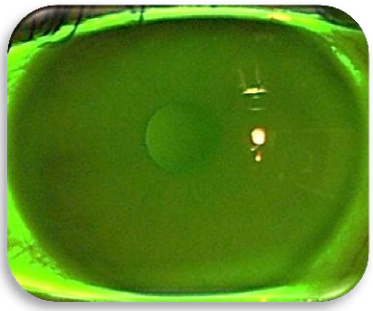
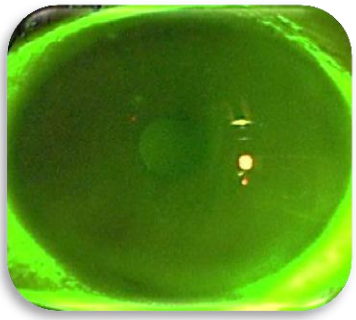
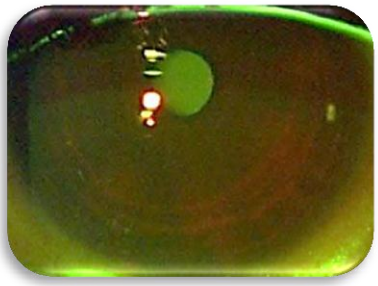
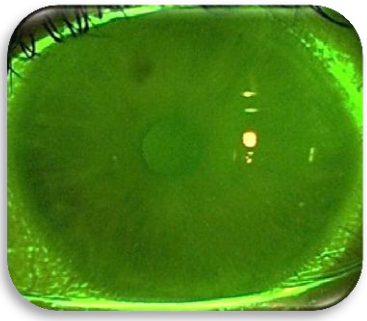
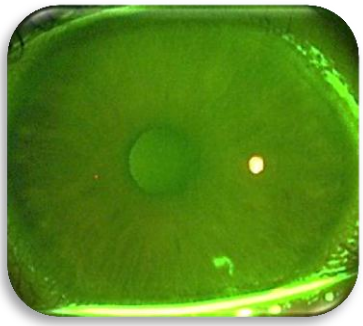
Subject 1 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 2 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 3 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 4 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**

Figure A12e Slit-lamp images with fluorescein pre and post CEC exposure to desiccating conditions for 60 minutes and after saline instillation in the right eyes of normal subjects 1-4.

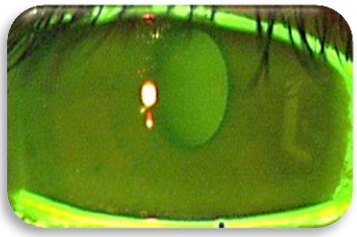
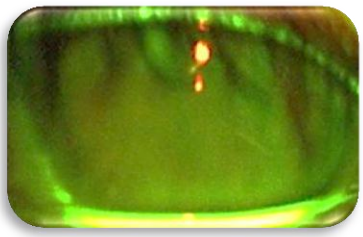
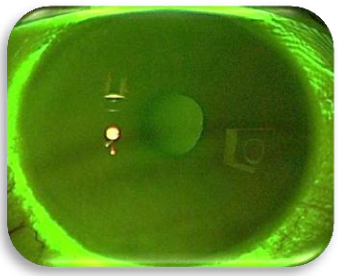
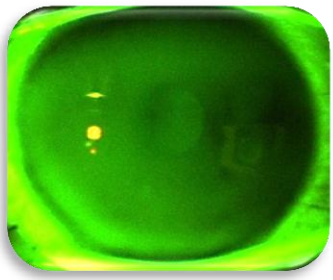
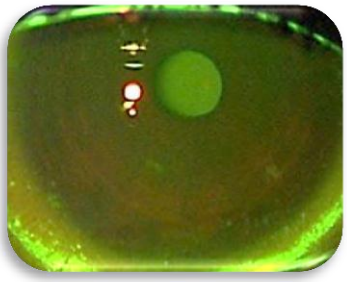
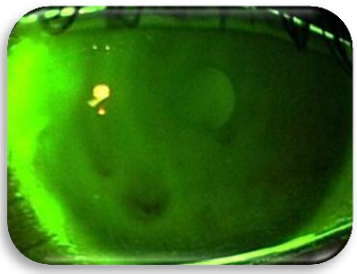
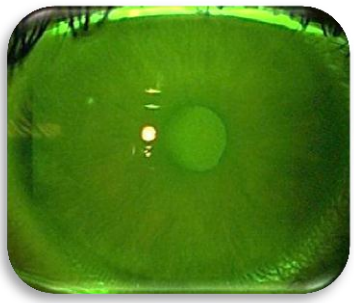
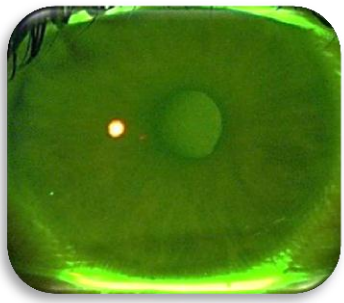
Subject 1 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 2 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 3 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 4 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**

Figure A12f Slit-lamp images with fluorescein pre and post CEC exposure to desiccating conditions for 60 minutes and after saline instillation in the left eyes of normal subjects1-4.

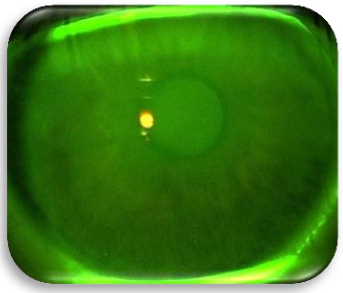
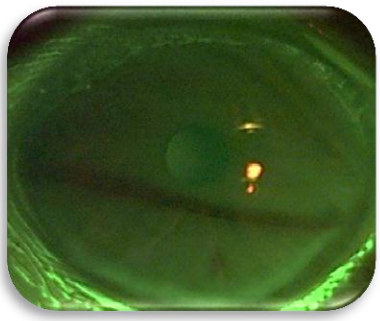
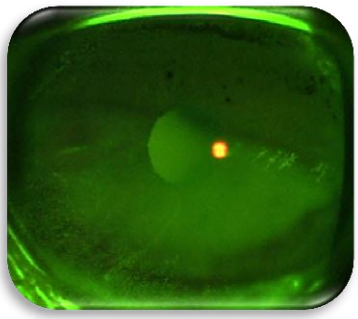
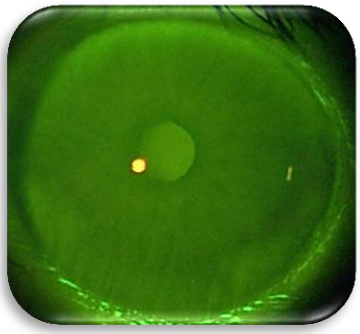
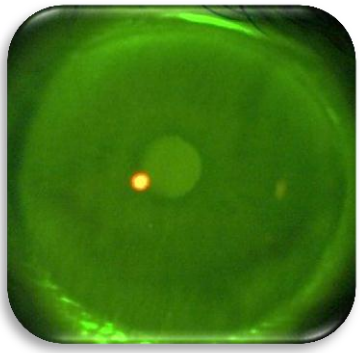
Subject 5 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 6 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 7 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 8 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**

Figure A12g Slit-lamp images with fluorescein pre and post CEC exposure to desiccating conditions for 60 minutes and after saline instillation in the right eyes of normal subjects 5-8.

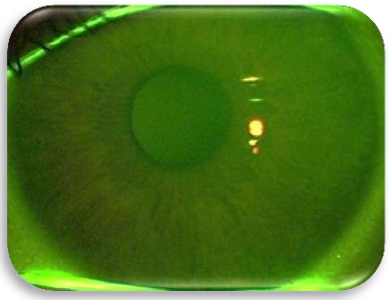
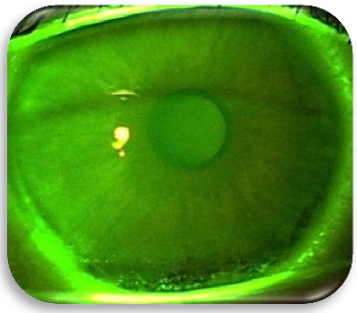
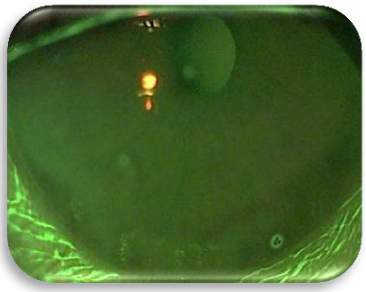
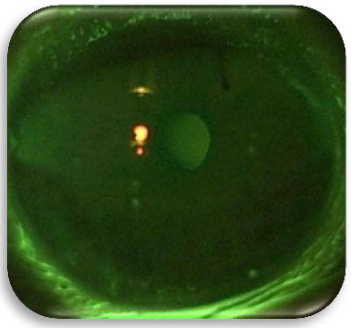
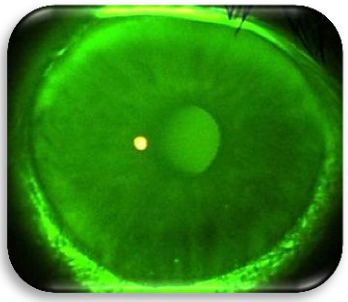
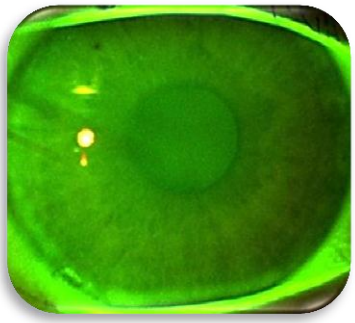
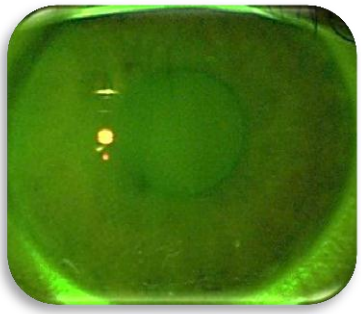
Subject 5 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 6 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 7 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 8 **Normal**		
	PRE-EXPOSURE	**POST-EXPOSURE**

Figure A12h Slit-lamp images with fluorescein pre and post CEC exposure to desiccating conditions for 60 minutes and after saline instillation in the left eyes of normal subjects 5-8.

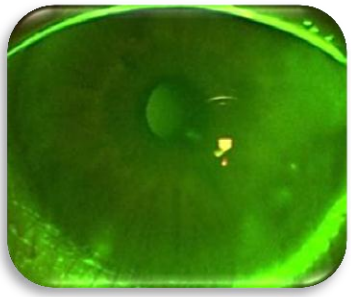
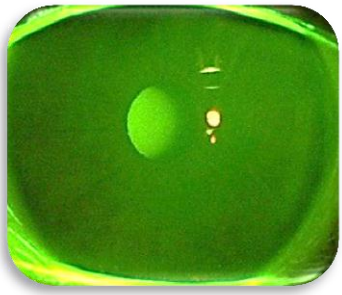
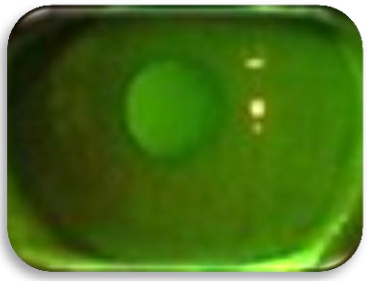
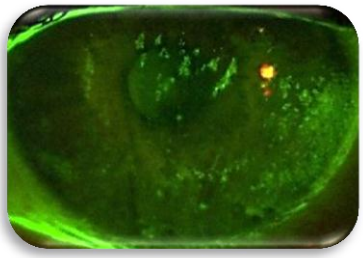
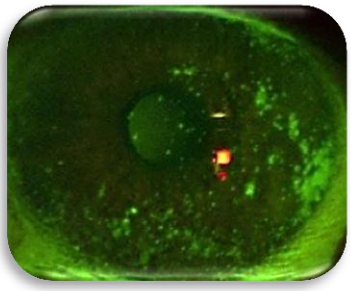
Subject 1 **DED**	 **PRE-EXPOSURE**	 **POST-EXPOSURE**
Subject 2 **DED**	 **PRE-EXPOSURE**	 **POST-EXPOSURE**
Subject 3 **DED (SjS)**	 **PRE-EXPOSURE**	 **POST-EXPOSURE**
Subject 4 **DED (SjS)**	 **PRE-EXPOSURE**	 **POST-EXPOSURE**

Figure A12i Slit-lamp images with fluorescein pre and post CEC exposure to desiccating conditions for 60 minutes and after saline instillation in the right eyes of DED subjects 1-4.

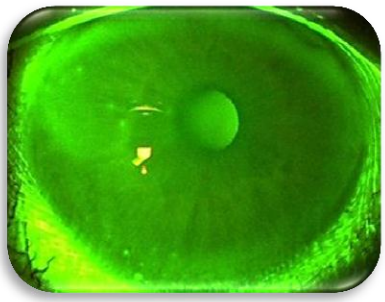
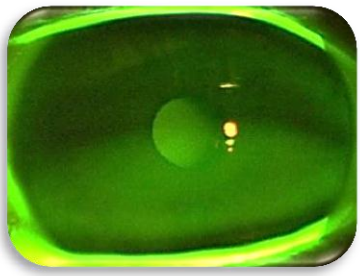
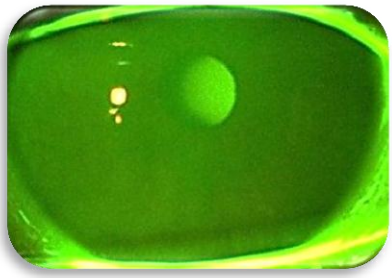
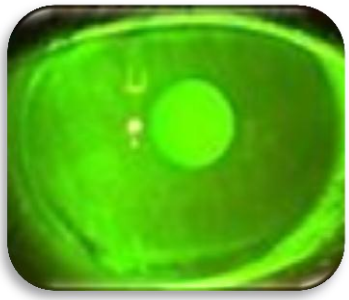
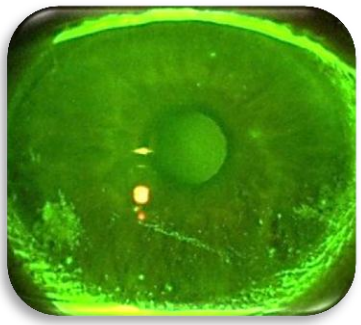
Subject 1 **DED**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 2 **DED**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 3 **DED (SjS)**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 4 **DED (SjS)**		
	PRE-EXPOSURE	**POST-EXPOSURE**

Figure A12j Slit-lamp images with fluorescein pre and post CEC exposure to desiccating conditions for 60 minutes and after saline instillation in the left eyes of DED subjects 1-4.

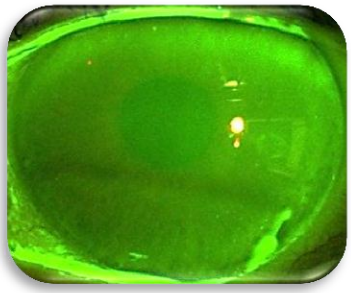
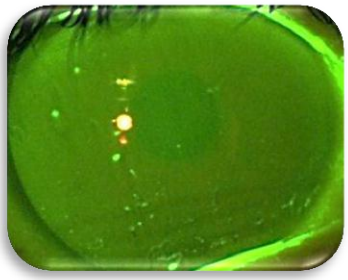
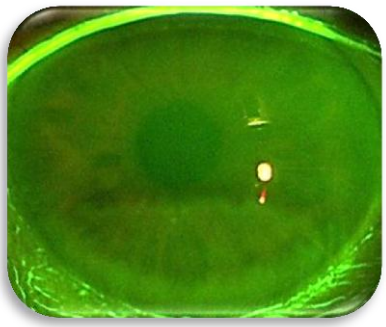
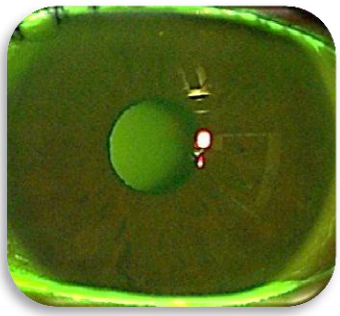
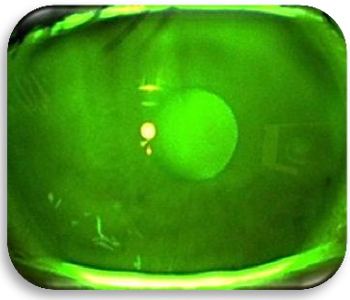
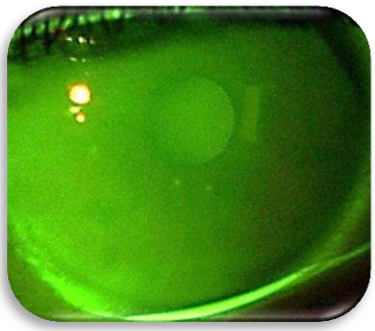
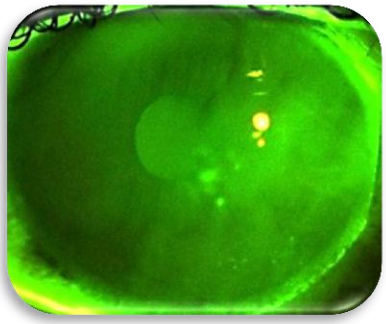
Subject 5 **DED (SjS)**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 6 **DED (SjS)**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 7 **DED**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 8 **DED**		
	PRE-EXPOSURE	**POST-EXPOSURE**

Figure A12k Slit-lamp images with fluorescein pre and post CEC exposure to desiccating conditions for 60 minutes and after saline instillation in the right eyes of DED subjects 5-8.

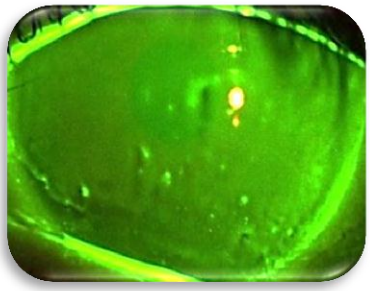
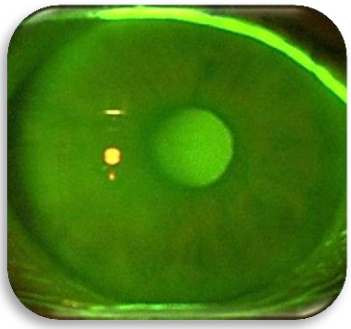
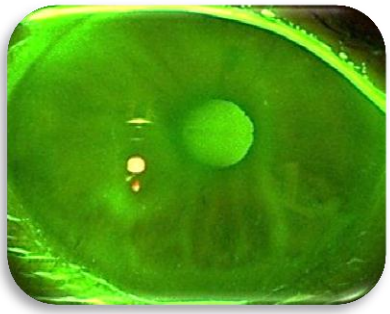
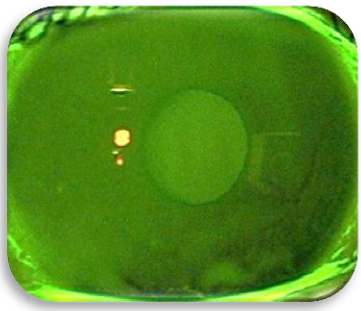
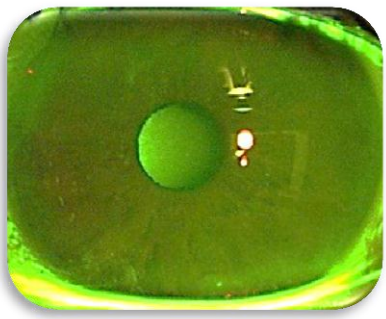
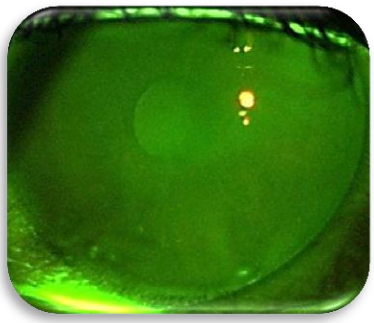
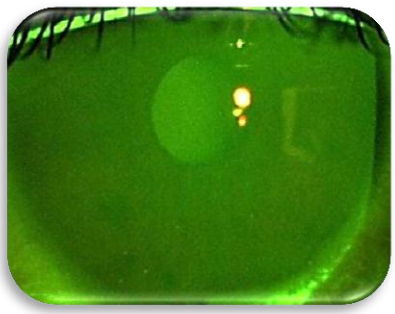
Subject 5 **DED (SjS)**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 6 **DED (SjS)**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 7 **DED**		
	PRE-EXPOSURE	**POST-EXPOSURE**
Subject 8 **DED**		
	PRE-EXPOSURE	**POST-EXPOSURE**

Figure A12l Slit-lamp images with fluorescein pre and post CEC exposure to desiccating conditions for 60 minutes and after saline instillation in the left eyes of DED subjects 5-8.

APPENDIX XIII: THE UTILITY OF THE TEARLAB® DEVICE AS A LABORATORY INSTRUMENT AND IN CLINICAL USE

A13.1 Introduction of tear osmolarity as a measure of dry eye disease

The purpose of this appendix is to appraise the reliability of the TearLab® osmometer and its utility in clinical use. Aspects of normal tear osmolarity values, diurnal variation and inter-eye differences are discussed in Chapter 1 (1.11 and 1.12) and Chapter 2 (2.7.4).

Tear osmolarity (tOsm) has been of interest since the 1940s when a balance between evaporation and tear flow was shown to determine its outcome (von Bahr, 1941). Mishima and Maurice (1961b) showed the importance of the meibomian oil in retarding evaporation and later, Gilbard et al., (1989) indicated that obstruction of the total complement of meibomian glands in a rabbit model led to a rise in tear film osmolarity. By this point a new technique for measuring tOsm was in use, the Clifton Nanolitre osmometer. This machine helped to establish that hyperosmolarity played an important role in inducing the corneal and conjunctival features of keratoconjunctivitis sicca (KCS) (Gilbard et al., 1978). Subsequent experiments showed a relationship between tear hyperosmolarity and Rose Bengal staining in dry eye disease (DED) (Gilbard and Farris, 1979) and a reduction in conjunctival goblet cell density (Gilbard et al., 1988). Experimentally, corneal epithelial cells in culture were morphologically altered following exposure to hyperosmolar solutions (Gilbard et al., 1984).

Tear osmolarity is now considered to be the best single measure of DED and has been proposed as the gold standard in diagnosis in DED (Farris, 1994; Lemp, 2011; Wolffsohn et al., 2017). Hyperosmolarity has been shown to be the primary cause of ocular surface damage and is a pivotal pathophysiological factor in both aqueous deficient dry eye (ADDE) and evaporative dry eye (EDE) (Bron et al., 2017).

A13.2 Types of osmometer

Various instruments have been used to measure osmolarity over the years, based on the principle that the osmolarity of a sample can be determined by the colligative properties of a solution, i.e. it is dependent on the number of dissolved particles in a solution but not on their size or weight.

A13.2.1 Depression of freezing point

Depression of freezing point osmometers use the principle that the total number of dissolved particles in a solution is directly proportional to its freezing point. The osmolarity is calculated from the depression in the freezing point and is the only method that measures concentration based solely on the number of dissolved particles in the sample. The benefit of these systems, in relation to the measurement of tOsm, is that a relatively

small tear sample is required, between 0.5-2µL, which reduces the risk of stimulating reflex tearing during sampling. Transfer of the sample from the eye to the instrument and the initial time elapsing during processing, can still be a potential source of error due to the occurrence of evaporation. Judging the point at which the melting point has been achieved is also subjective and can also be a problem. There are three commercially available instruments that utilise this technology: the Advanced® Micro-Osmometer (Advanced Instruments, MA, USA), the Otago Osmometer (Otago, Dunedin, New Zealand) and the Clifton Nanolitre Osmometer (Technical Physics of Hartford, NY, USA).

A13.2.2 Vapour pressure

In a similar way, the vapour pressure of a solution is a measure of its osmolarity since vapour pressure is directly proportional to the number of dissolved particles in the solution. The only commercially available instrument is the Wescor vapour pressure osmometer (VAPRO, Wescor, Inc., UT, USA). The sample here is analysed without the need to change its physical state, (as with depression of freezing point osmometers) which, increases the reliability of results, but the technique does require a larger sample to operate, (10µL), which is often unachievable from DED patients who by the very nature of their disease, in aqueous-deficient DED, will have a reduced tear supply.

A13.2.3 Electrical impedance

Lastly, osmolarity can also be measured as a function of electrical impedance. This method measures the electrical conductivity of a tear sample, and is dependent on the number of charged particles in the solution. A change in the composition or concentration of the ions in a sample will alter the conductivity and is an indirect function of osmolarity.

Currently available machines include the TearLab® Osmolarity System (TearLab Corporation, CA, USA) and the i-Pen® (I-MED Pharma Inc., Montreal, QC). These machines require the smallest samples of all the osmometers (50nL for the TearLab®) and allow an almost instantaneous analysis of the electrical properties of the solution, which can be converted into an osmolarity value.

A13.3 Correlation between TearLab and other osmometers

Results obtained from the TearLab® osmometer have been reported to compare well with commercially available devices considered to be the gold standard in the measurement of osmolarity in the laboratory by several authors (Versura et al., 2010; Versura and Campos, 2013; Tomlinson et al., 2010; Rocha et al., 2017).

Tomlinson and colleagues compared the osmolarity of tears collected from a series of 15 DED subjects with that of 21 normal controls, evaluated with both the TearLab® system and the Clifton Nanoliter osmometer (Tomlinson et al., 2010). A good agreement between the two instruments was demonstrated ($r = 0.904$; $p = 0.006$) and the same cut-off level calculated between the two populations (316 mOsm/L).

In a further study, the repeatability of sampling from 20 normal subjects using the TearLab® was tested. The TearLab® showed a good test-retest correlation between repeated measurements ($r = 0.8$, $P < 0.05$). Additionally in this study, measurements were taken with the TearLab® and compared with those using the Wescor vapour pressure osmometer, in 52 healthy participants (who, incidentally, would be capable of supplying sufficient tear volume for this technique). The mean tOsm in the subjects, using the TearLab®, was 299.2 ± 10.3 mOsm/L and with the Wescor instrument 298.4 ±10.0 mOsm/L. These two results were not significantly different from one another ($P < 0.05$) (Gokhale et al., 2013).

Later an *in vitro* study examined the comparison of results on contrived tear solutions designed to represent normal (297 mOsm/L), moderate DED (342 mOsm/L) and severe DED (383 mOsm/L) between the TearLab® and Wescor vapour pressure devices. The TearLab® osmometer reported a linear regression of $r^2 = 0.96$ and the Wescor vapour pressure $r^2 = 0.98$. The authors concluded that these two machines performed similarly in their ability to determine the osmolarity of these contrived solutions of known values (Rocha et al., 2017).

A13.4 Precision and accuracy and of the TearLab® osmometer

Understanding the reliability of an instrument is essential for confidence in its clinical use. The coefficient of variation (CV) is often reported as a measure of relative variability. The Wescor vapour pressure osmometer, which requires a higher volume than the TearLab® machine, is regarded as a laboratory standard. The CVs from studies analysing the variation of the TearLab® and Wescor osmometers *in vivo* ranged from 1% to 4.1% (Eperjesi et al., 2012; Nolfi and Caffrey, 2017; Rocha et al., 2017; Reis et al., 2017). The CV values stated above encapsulate those reported by the manufacturers of the TearLab® machine of 1.5% (US FDA. k083184, TearLab Osmolarity System. April 23, 2009).

Concerning the accuracy of the instrument, a more recent study by Nolfi and Caffrey (2017) measured the analytical performance of the TearLab® machine using Quality Control (QC) standard solutions, used to calibrate the machines, over a period of two months. An average osmolarity of 335.8 mOsm/L ± 4.2 (target of 338 mOsm/L) was documented, yielding an accuracy for the TearLab® of 99.4%. The mean osmolarity of the QC solutions analysed by the two TearLab® machines used in this thesis was 336.9mOsm/L ± 1.15, yielding an accuracy of 99.7%.

Positive predictive values (PPV) provide an index of performance which relates the proportion of positive results in a diagnostic test to the number of true positive results. This value, along with sensitivity and specificity, are used to confirm the utility of a

measurement value for diagnostic purposes. Several different index values have been documented in previous studies using the TearLab® that resulted in a high PPV level. Tomlinson et al., (2010) cited a cut-off of 316 mOsm/L with a PPV of 85%, sensitivity of 73% and specificity of 90%; the tOsm results obtained in this study were similar to those reported by the same group in a previous meta-analysis of osmolarity values in DED subjects (Tomlinson et al., 2006). The group also reported that values for the TearLab® correlated well with those from the Clifton Nanoliter osmometer using a cut-off of 317 mOsm/L, which yielded a PPV of 65%, sensitivity of 73% and specificity 71% (Tomlinson et al., 2006). Versura et al., (2010) cited a cut-off figure of 305 mOsm/L that yielded a PPV of 98.4%, sensitivity of 44% and specificity of 96% and Khanal et al., (2008) reported a cut-off figure of >317 mOsm/L and a PPV of 86%, sensitivity of 78% and specificity of 78%. For the current thesis a cut-off of 308 mOsm/L was chosen to distinguish between normal and DED subjects based on the Lemp et al., (2010) paper which concluded that this figure provided the most sensitive threshold to distinguish between normal and mild/moderate DED. This was based on data from a multicentre, ten site study, of 314 subjects, that evaluated the clinical utility of tOsm for assessing DED severity. The most specific cut-off value from this study was 315 mOsm/L for tOsm exhibiting 73% sensitivity and 92% specificity.

A13.5 Sources of TearLab® measurement variation in clinical use

Variation of measurements using the TearLab® osmometer may arise from an assortment of factors, including instrument and biological variation, collection technique and environment.

Khanal et al., (2012) stated that the variability of the TearLab® measures could be related to the machine itself. In their study eight occasions out of approximately 160 produced either no readings or displayed an ‘out-of-range’ report. The TearLab® machine will only register osmolarity measures between 270-400 mOsm/L. The machines used for this thesis were specially adapted in collaboration with the manufacturers to allow the out-of-range values to be shown. In the event, no ‘out-of-range’ values were registered by the machines in all of the four studies reported in this thesis. Khanal et al., (2013) also stated anecdotally that the readings that took longer to obtain produced higher results. In this thesis as a way of minimising such errors, any readings that registered a discomfort score of three, indicating that there may have been more difficulty in obtaining the sample, were discarded, as a precaution.

In the study of Nolfi et al., (2017) there was no evidence of analytical outliers using the TearLab®, the authors asserting that any elevated values obtained with this osmometer, both in this study and others (Bunya et al., 2015; Szczesna-Iskander 2016) were an

essential aspect of tOsm variation in the early stages or in mild DED. These extreme data points, rather than being outliers should be considered important signals that indicate an unstable ocular surface before traditional clinical signs have revealed the disease. The authors concluded that “even if a perfectly accurate and precise osmometer existed, the specificity of tear osmolarity would be limited to the 90% range that is reported elsewhere, due to the inherent nature of the marker” and as such osmolarity measured using the TearLab® machine is still considered a valid measure of DED in clinics and experimental studies alike.

The positioning of the pen tip of the handset when collecting tear samples has been cited as a potential source for variability (Wunderlich et al., 2011). The authors designed an *in vitro* study whereby the angle of the TearLab® pen was changed between 70 - 120° with a liquid meniscus being created by applying a Ringer’s solution at the junction of two clean glass plates that had been placed on top of each other and displaced sideways. The tOsm differed by 8.9 mOsm/L between 90 to 120° from 314.4 ± 7 to 323.3 ± 5.4 respectively. Similarly, Szczesna-Iskander (2016) observed that there was less variability in tOsm values when a right-handed examiner took readings from the left eye and attributed this to increased comfort and more consistent and adequate positioning of the test card in obtaining the sample. In this thesis every attempt was made to standardise conditions when measuring patients, the same practitioner (right-handed) was responsible for taking all samples at each visit and had received training on the technique of tear sampling from a TearLab® representative as well as practising the technique within the supervisory team for at least a year before patient recruitment. In fact, as documented by the Khanal et al., (2012) study there was very low intra-observer variability when taking samples with the TearLab® machine.

Environmental factors can contribute to errors in tOsm measurements. Reduced relative humidity can influence osmolarity by increasing evaporation from the ocular surface or inducing reflex tearing. Tear meniscus has been shown to be reduced in presence of increased airflow exposure making sampling more difficult (Koh et al., 2012), which can potentially influence results further. Temperature can also affect the outcome of impedance measures, with a difference of approximately 2% being reported for every change in degree (Calles, 1990). Although *in vitro* studies measuring the osmolarity of biologic fluids with the help of electrical impedance have shown that a temperature variation between 20°C and 40°C led to only a minimal change of approximately 1.8% per degree centigrade (Fouke et al., 1987). The TearLab® osmometer uses temperature-compensated impedance calculations to measure tOsm. Consequently, the manufacturers recommend that the analytical performance of the machines is tested on each day of data collection by calibrating with a National Institute of Standards and Technology traceable sodium chloride QC solution with a target of 338 mOsm/L (330-346

mOsm/L). In the set of experiments for this thesis, two TearLab® machines were employed; one inside and one outside the controlled environment chamber (CEC). The temperature inside the CEC was maintained between 21-23°C during experimentation, whilst the machine outside was situated in uncontrolled 'clinic' conditions where the temperature ranged from 17-26°C. Both machines passed the daily calibration procedures using the manufacturers' electronic test cards and saline control solution.

Many of the experiments that report a variability of TearLab® results or that data from the machine should be viewed cautiously due to outliers were conducted in uncontrolled environmental clinic conditions (Khanal et al., 2013; Szalai et al., 2012; Alves et al., 2013; Amparo et al., 2014; Bunya et al., 2015; Baenninger et al., 2018) and as such did not control for these potentially confounding elements. The experiments in this thesis engaged in the standardisation of environmental conditions in an attempt to minimise any effects on the osmolarity of collected samples.

A13.6 Advantages of TearLab® sampling system

Of the earlier available osmometers, the Wescor vapour pressure osmometer despite its ease of use was of little practical value in the diagnosis of DED because of its high sample volume requirement exceeding that likely to be available in ADDE. The Clifton Nanoliter osmometer, of equal reliability and requiring a smaller and more relevant sample size, made an important contribution to our understanding of tOsm behaviour in normal and dry eyes, but because of its high cost and requirement for significant technical expertise in processing samples, was relegated to use as a research tool. Even here, the volume requirement was such that there was some risk of stimulating reflex tear secretion during collection, with possible dilution of the sample and similarly, a risk of evaporative loss both during tear collection and processing, with possible concentration of the sample. With the TearLab® osmometer, the tear sample is aspirated rapidly from the tear meniscus (within about 2-3 seconds) by capillary attraction and analysed almost immediately using lab-on-a-chip technology. An osmolarity readout is provided within a couple of minutes. Because of the small volume requirement and speed of sampling, the risk of reflex tearing is greatly reduced, although ease of sampling does vary on an individual basis. The lab-on-chip technology which acts as both the analytical system and collection device eliminates the evaporative element and source of potential error of previous machines during transfer of the samples (Nelson and Wright, 1986) and avoids the need for capillary tubes or acetate discs for tear sampling, improving the reliability of the procedure (Versura et al., 2010). The TearLab® has been reported to have increased utility especially in subjects with a reduced tear lake, since sufficient samples could be obtained using the

TearLab® that could not be collected, even after repeated attempts, using the vapour pressure osmometry method (Gokhale et al. 2013).

New instrumentation, such as the i-Pen®, has been designed to measure the osmolarity of the tears that bathe the orbital tissues such as the palpebral conjunctiva and represent tOsm levels occurring at the tissue level. The utility of this osmometer compared to other commercially available devices has been investigated *in vitro* and *in vivo* by several groups (Reis et al., 2017; Rocha et al., 2017; Nolfi et al., 2017). These studies concluded that the i-Pen often reported random values and was unable to reproduce the expected range of normal tOsm values in subjects without DED. The latter function is essential, to provide a criterion of hyperosmolarity. However, the endeavour to identify osmolarity at the ocular surface rather than in the tear meniscus is important, since it has previously been reported in modelling papers (Gaffney et al., 2010) that tOsm sampled at the meniscus does not necessarily reflect those values occurring at other places on the ocular surface. The method of sampling used with the i-Pen however, is likely to induce more reflex tearing due to contact with the ocular surface during sampling. This, along with a lack of daily calibration could lead to unreliable results (Nolfi et al., 2017) and may be responsible for an absence of correlation between this machine, the TearLab® and Wescor vapour pressure osmometers (Rocha et al., 2017).

A13.7 Clinical utility of the TearLab® osmometer

Tear film osmolarity has been shown to be the single best marker of disease severity (Sullivan et al., 2010; Versura et al., 2010; Lemp et al., 2011; Utine et al., 2011; Jacobi et al., 2011), with the ability to discriminate between normal controls and patients with mild, moderate or severe DED. Studies have reported that tOsm reflects objective signs of inflammation as well as correlating significantly with subjective discomfort symptoms (VanDerMaid et al., 2012). High levels of inflammatory mediators detected in ocular surface disease have also been estimated with the use of tOsm values obtained using the TearLab® device (Versura et al., 2011), further justifying its use in the clinical setting.

The TearLab® osmometer allows tOsm to be measured quickly during an examination and has facilitated investigation of tear-related conditions, including both the diagnosis and effectiveness of treatments. Clinical trials have reported a reduction in tOsm towards normal values following intervention with a variety of treatments, including hyaluronic acid based tear substitutes (Benelli et al., 2010), Cyclosporin A (Sullivan et al., 2010) and autologous serum (Na and Kim, 2012). Jacobi et al., (2011) reported that testing the osmolarity of the tear film was an effective objective test for DED diagnosis, showing a significantly higher tOsm in patients with severe keratoconjunctivitis sicca compared with the healthy controls (Jacobi et al., 2011). A positive correlation was reported between

tOsm and severity, using the TearLab® osmometer, comparing patients with primary Sjögren syndrome (SjS) with control subjects (Utine et al., 2011). More recently tOsm was shown to reflect both objective signs and symptom severity in SjS patients (Kim et al., 2017).

Along with its extensive use in the field of dry eye, the TearLab® osmometer has also been incorporated into study designs in other areas of Ophthalmology. Tear osmolarity has been used as an outcome measure of ocular surface health following the use of Glaucoma pressure-lowering eye drops (Labbe et al., 2012; Januleviciene et al., 2012).

A13.8 Conclusion

The TearLab® instrument provides as precise a measure of osmolarity of a fluid mixture as instruments employed to measure osmolarity in larger volume. The risk of dilution of a tear sample due to reflex tearing is minimised by rapid sampling and there is no risk of evaporative loss during processing and analysis. Possibly some of the unwanted variation referred to in the literature arises from a lack of standardisation of the sampling technique during use, the employment of multiple investigators and a lack of environmental standardisation.

APPENDIX XIV: SUPPORTING PUBLICATIONS