Aqueous tear assessment in dogs: impact of cephalic conformation, inter-test correlations and test-retest repeatability

Hellen Bolzani[a], Arianne P. Oriá[a*], Ana Claudia S. Raposo[a], Lionel Sebbag[b]

[a] School of Veterinary Medicine and Zootechny, Federal University of Bahia, UFBA, 500, Avenida Adhemar de Barros, Salvador 40170-110, Brazil

[b] Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Iowa State University, Ames, Iowa 50011, USA

∗ Corresponding author: Dr. Arianne P. Oriá

Tel.: 55 71 32836749

Fax: 55 71 32836730

E-mail: arianneoria@ufba.br

Running title: Aqueous tear assessment in dogs

Abstract

Objective – To characterize diagnostic findings, test-retest repeatability, and correlations among lacrimal tests in dogs of diverse cephalic conformations.

Animal studied – Fifty healthy dogs (25 brachycephalic, 25 non-brachycephalic).

Procedures – A series of diagnostics were performed in each dog, allowing for a 10min-interval between tests and repeating each test 24h later under similar conditions: corneal tactile sensation (CTS), strip meniscometry test (SMT), phenol red thread test (PRTT), endodontic absorbent paper point tear test (EAPPTT), Schirmer tear test-1 without (STT-1) or with nasolacrimal stimulation (NL-STT1), and Schirmer tear test-2 (STT-2).

Results – Mean ± SD test values were lower in brachycephalic *vs.* non-brachycephalic dogs (except for SMT; 7.4±2.0 mm/5s *vs.* 7.3±2.4 mm/5s), with statistically significant differences noted for CTS (1.8±0.5 cm *vs.* 3.4±0.8 cm), PRTT (37.2±4.0 mm/15s *vs.* 41.1±5.5 mm/15s), STT-1 (20.1±3.4 mm/min *vs.* 23.3 ±5.7 mm/min), STT-2 (13.0 ± 3.4 mm/min vs 16.9 ± 3.9 mm/min), and NL-STT1 (23.2±3.6 mm/min *vs.* 27.1±5.4 mm/min), and non-significant differences for EAPPTT (16.6±2.7 mm/15s *vs.* 17.5±2.9 mm/15s). Nasolacrimal stimulation increased STT-1 values by 18% on average. Correlations among tests were generally weak to moderate ($r<0.70$) except for a strong correlation between STT-1 and NL-STT1 ($r=0.83$, $P<0.001$). Tests reliability was good although test-retest repeatability was generally poor to moderate, as depicted by low intraclass correlation coefficients (ICC≤0.75) and wide 95% limits of agreement, except for CTS (ICC=0.91).

Conclusions – Corneal sensitivity and aqueous tear secretion are lower in brachycephalic dogs. A comprehensive assessment of the ocular surface requires the combination of several diagnostic tests. The nasolacrimal reflex may provide a useful diagnostic and therapeutic tool in dogs.

KEYWORDS: Canine, Corneal sensitivity, Keratoconjunctivitis sicca, Nasolacrimal reflex, Tear production, Reliability

INTRODUCTION

The measurement of tear production is an important step in assessing the ocular surface health in dogs and other species. The Schirmer tear test-1 (STT-1), developed in 1903 by the German ophthalmologist Otto Schirmer,[1] has long been considered the gold-standard tool for measuring the aqueous component of the tear film. Other lacrimal tests commonly reported in dogs include the STT performed following topical anesthesia (STT-2) and the phenol red thread test (PRTT).[2-4] Tear assays are constantly evolving, and two additional tools were recently proposed for lacrimal testing in veterinary species: the endodontic absorbent paper point tear test (EAPPTT), and the strip meniscometry test (SMT).[5,6] Each diagnostic test has its advantages and limitations in assessing tear production in dogs. For instance, the testing duration is the shortest with SMT (5 sec), providing a minimally invasive tool for discriminating tear-deficient from normal eyes in clinical practice.[6] In contrast, STT requires 60 seconds of testing and can cause more irritation, albeit this longer duration provides invaluable insight into different components of tearing (basal, reflex) and the neural pathway responsible for lacrimation.[7] Indeed, the Schirmer strip stimulates the afferent corneal and conjunctival nerves, activating the efferent parasympathetic and sympathetic nerves that innervate the lacrimal gland.[8] In fact, the afferent trigeminal nerve can be stimulated in locations other than the ocular surface, as demonstrated by the nasolacrimal reflex. The nasolacrimal reflex quantifies tear secretion with STT while causing sensory stimulation of the nasal mucosa.[9]

Test–retest repeatability, or the properties of a measurement tool evaluated twice on separate occasions, is an important factor to consider when using a diagnostic test in clinical practice. Indeed, a poor test repeatability can hinder the clinician's ability to evaluate changes attributable to disease progression or therapeutic effects. This is the case for selected diagnostic tests in humans[10] and cats.[11] In dogs, however, the repeatability of ocular surface diagnostics is not well studied to date, except for meibometry[12] and tear osmometry,[13] despite the high prevalence of dry eye disease and other ocular

surface conditions in this species.[14] Ideally, such investigation should account for the variability in cephalic conformation seen in canine breeds, as differences in anatomical conformation (*e.g.* macropalpebral fissure, trichiasis) and corneal innervation[15] between brachycephalic and non-brachycephalic dogs could theoretically affect the repeatability of lacrimal tests.

The present study describes the diagnostic findings, test-retest repeatability, and correlations among lacrimal tests in canine subjects of diverse cephalic conformations. A second study objective is focused on the nasolacrimal reflex, a promising tool that could have diagnostic and therapeutic implications in dogs, as shown in multiple studies in humans[9,16-21] and a single report in cats.[22]

MATERIAL AND METHODS

1. Animals

Fifty client-owned dogs were enrolled in the study, all confirmed to be ophthalmoscopically healthy with adequate tear production based on slit lamp biomicroscopy (SL-17, Kowa, Torrance, United States), indirect ophthalmoscopy (Eyetec, São Carlos, Brazil), tonometry (TonoVet, Icare, Vantaa, Finland), and Schirmer tear test-1 ≥ 15 mm/min.

The study population comprised 24 males and 26 females, aged between 4 months and 9 years (4.23 ± 2.4 years). Cephalic index (CI) was determined in each dog by measuring the skull's length and width as previously described,[23] and this parameter was used to characterize each dog as brachycephalic (n = 25) or non-brachycephalic (n = 25) per Evans and De Lahunta.[24] Informed consent was obtained from all owners, and the study was approved by the Ethics Committee on Animal Experimentation of the Federal University of Bahia.

2. Experimental design

All experiments were performed in the morning hours (8-12) by a single examiner (HB) in the same examination room, with ambient temperature and humidity recorded daily. The following diagnostic tests were performed in both eyes of each dog in the specific order described below, ensuring a minimum of 10min interval between successive tests to allow the tear fluid to replenish.[23] Further, all tests were repeated 24h later in all patients to assess test-retest repeatability.

- **Corneal tactile sensation (CTS):** A Cochet-Bonnet aesthesiometer (Luneau Ophtalmologie, Chartres, France) with a 0.12-mm diameter monofilament was used to evaluate the corneal sensitivity in each eye. Starting at a filament length of 6 cm, the nylon fiber was held perpendicular to the ocular surface and advanced toward the central cornea until a slight bend in the fiber was noted (**Figure 1A**). The filament was shortened in increments of 0.5 cm, and CTS was recorded as the length (in cm) that elicited consistent blink reflex in at least 3 out of 5 attempts. Corneal sensitivity is reported in cm for consistency with recent studies in veterinary[25,26] and human[27] literature, and because the actual pressure applied onto the cornea (g/mm^2) varies greatly when the same examiner uses the same filament length.[28] However, to allow for comparisons with other studies, the data is also reported in g/mm^2 in Table 1 using the conversion table for 'new' nylon filament.[28]
- **Strip meniscometry test (SMT):** A standard SMT strip (i-Tear® Test, I-Med Pharma Inc., Dollard-des-Ormeaux, Canada) was placed in contact with the central lower tear meniscus for 5 seconds, ensuring the eyelid position remained neutral (*i.e.* no manual eversion) and avoiding contact with the corneo-conjunctival surface.[6] Tears are absorbed via capillary action into the central channel of the strip, and measurements are recorded in mm/5s (**Figure 1B**).
- **Phenol red thread test (PRTT):** The lower eyelid was gently everted, and the bent portion of the thread (Zone-Quick™, Oasis Medical, Glendora, CA, USA) was placed into the lateral lower

conjunctival fornix for 15 seconds. The wetted portion of the thread was measured with a ruler, a process facilitated by a color change of the thread from yellow to red, and results were recorded in mm/15s (**Figure 1C**).

- **Endodontic absorbent paper point tear test (EAPPTT):** A paper point size #30 (Roeko, Langenau, Germany) was inserted in the lower conjunctival fornix for 15 seconds. The wetted portion of the paper point was measured with a ruler, and results were recorded in mm/15s (**Figure 1D**).
- **Schirmer tear test-1 (STT-1):** A standard Schirmer strip (Tear Flo™ ophthalmic strips, Oasis Medical, Glendora, CA, USA) was placed in the lateral lower conjunctival fornix for 60 seconds, and tear production was recorded in mm/min (**Figure 1E**). Results were recorded as 35 mm/min if the entire strip was wetted before the full minute had elapsed.
- **Schirmer tear test-1 with nasolacrimal stimulation (NL-STT1):** A standard Schirmer strip was placed in the lateral lower conjunctival fornix of each eye, followed immediately by placing a cotton ball soaked with 70% alcohol in front of (by not touching) the animal's nostrils (**Figure 1F**). Olfactory stimulation was sustained throughout the Schirmer testing (1 minute), and results were recorded in mm/min.
- **Schirmer tear test-2 (STT-2):** One drop of proxymetacaine ophthalmic solution (Anestalcon®, Alcon, São Paulo, Brazil) was administered in both eyes. Five minutes later, a standard Schirmer strip was placed in each eye as described for STT-1, and results were recorded in mm/min.

3. Data analysis

Normality of the data was assessed with the Shapiro–Wilk test, and results from right and left eyes were compared by means of paired t-tests. Since data was normally distributed ($P \geq 0.082$), results are presented as mean ± standard deviation (range). Further, since no significant differences were noted between eyes

for any diagnostic test ($P \geq 0.088$), only data from the right eye was used for subsequent analyses as averaging both eyes could potentially confound correlation and repeatability analyses.[11,29]

Differences in age and diagnostic tests results between brachycephalic and non-brachycepahlic dogs were assessed with the Student's t-test. The Pearson's correlation test was used to assess for correlations among diagnostic tests and for potential association between cephalic index and each diagnostic test. Results of Pearson's tests were interpreted following guidelines described by Campbell and Swinscow[30]: very weak (0-0.19), weak (0.2-0.39), moderate (0.40-0.59), strong (0.6-0.79) and very strong (0.8-1.0). A paired t-test was used to assess differences between STT-1 and STT-2, as well as STT-1 and NL-STT1. The reliability of diagnostic tests (test-retest repeatability) was assessed with 3 complementary tools: (i) Paired t-test for measurements obtained on Day 1 *vs.* Day 2, assessing for potential systematic bias across both sessions; (ii) Intraclass correlation coefficients (ICCs, two-way model, single rater type) and their 95% confidence interval, obtained with MedCalc version 19.0.7 (MedCalc Software, Mariakerke, Belgium), assessing for absolute agreement between both sessions; and (iii) Bland-Altman plots,[31] graphically displaying differences between test-retest measures and the associated 95% limits of agreement (LoA). Results of ICCs were interpreted as representing poor (<0.40), moderate (0.40-0.75), or good (> 0.75) test reliability.[32] Except for ICCs, statistical analyses were performed with SigmaPlot 14.0 (Systat Software, Inc., San Jose, CA) and values $P < 0.05$ were considered statistically significant.

RESULTS

Throughout the entire study duration (10 months), ambient temperature and humidity varied from 24-30ºC and 40-70%, respectively; however, median (range) differences in temperature and humidity from one examination day to another (for the same patients) were only 1ºC (0-2ºC) and 3% (1-5%), respectively.

1. Brachycephalic and non-brachycephalic dogs

Brachycephalic dogs (n = 25) had a CI ranging from 90-120 (99.76 ± 6.93), and were comprised of 11 males and 14 females (4 males/11females were neutered) including 12 Shih-Tzus, 6 French Bulldogs, 6 Pugs, and 1 English Bulldog. Non-brachycephalic dogs (n = 25) had a CI ranging from 46-78 (57.32 ± 9.26), and were comprised of 13 males and 12 females (5 males/5 females were neutered) including 7 Dachshunds, 5 Belgian Malinois, 5 German Shepherds, 4 Labrador Retrievers, 2 Rottweilers, 1 Doberman Pinscher, and 1 Border Collie. There was no statistical difference (P = 0.985) between the age of brachycephalic (4.3 ± 2.2 years, 0.3-8.0 years) or non-brachycephalic dogs (4.2 ± 2.6 years, 0.3-9.0 years).

Results of ocular surface diagnostics are described in **Table 1**. Except for SMT, mean values of all diagnostic tests were lower in brachycephalic compared to non-brachycephalic dogs, with statistically significant differences noted for CTS (1.8 ± 0.5 cm *vs.* 3.4 ± 0.8 cm, respectively; $P < 0.001$), PRTT (37.2 ± 4.0 mm/15s *vs.* 41.1 ± 5.5 mm/15s, respectively; $P < 0.001$), STT-1 (20.1 ± 3.4 mm/min *vs.* 23.3 ± 5.7 mm/min, respectively; $P < 0.001$), STT-2 (13.0 ± 3.4 mm/min vs 16.9 ± 3.9 mm/min; $P < 0.001$), and NL-STT1 (23.2 ± 3.6 mm/min *vs.* 27.1 ± 5.4 mm/min, respectively; $P < 0.001$), and non-significant differences noted for EAPPTT (16.6 ± 2.7 mm/15s *vs.* 17.5 ± 2.9 mm/15s, respectively; $P = 0.136$).

2. Schirmer tear test and nasal neurostimulation

Tear production was significantly greater in unanesthetized eyes (STT-1) compared to anesthetized eyes (STT-2) in all dogs (21.7 ± 4.9 mm/min *vs.* 14.9 ± 4.1 mm/min; $P < 0.001$). Further, nasal neurostimulation (NL-STT1) achieved significantly higher tear measurements compared to standard STT-1 in all dogs (25.1 ± 4.9 mm/min *vs.* 21.7 ± 4.9 mm/min; $P < 0.001$). Mean ± SD (range) percent increase in tear production achieved with nasal neurostimulation, as obtained with the ratio (NL-STT1 – STT1)/(STT1), was 16 ±

11% (-8 to 38%) in brachycephalic dogs, 19 ± 19% (-18 to 113%) in non-brachycephalic dogs, and 18 ± 16% (-18 to 113 %) in all dogs combined.

3. Test-retest repeatability

The reliability of ocular surface diagnostics and the agreement in measurements obtained at two separate sessions are summarized in **Table 2**. Except for EAPPTT and STT-2 in brachycephalic dogs, the reliability of ocular diagnostics was adequate in all dogs (Pearson's correlation tests, $P \leq 0.020$). This finding, along with the lack of significant differences between measurements obtained in two separate days (paired t-tests, $P \geq 0.051$), supports the lack of systematic bias in measurements obtained in both sessions. However, the agreement between measurements was variable among ocular diagnostic tests. Although nuances were noted for brachycephalic and non-brachycephalic breeds (**Table 2**), the test-retest agreement in all dogs was generally poor (ICC < 0.5) for EAPPTT, moderate (ICC 0.5-0.75) for SMT, PRTT, STT-1, STT-2 and NL-STT1, and good (ICC > 0.75) for CTS. This variable test-retest repeatability is depicted graphically for each diagnostic test using Bland-Altman plots (**Figure 2**).

4. Inter-tests correlations

Cephalic index – CI had a strong negative correlation with CTS (r = -0.76; $P < 0.001$), as well as a weak negative correlation with PRTT (r = -0.35; $P < 0.01$), STT-2 (r = -0.43; $P < 0.001$), and NL–STT1 (r = -0.27; $P = 0.007$)(**Figure 3**).

Ocular diagnostic tests – Correlations among diagnostic tests are summarized in **Table 3**. Of note, most inter-tests correlations (when present) were generally weak to moderate (r < 0.70) except for a strong correlation between STT-1 and NL-STT1 in all cases (r ≥ 0.80; $P < 0.001$). Further, diagnostic tests that presumably capture 'basal' tearing (SMT, PRT, EAPPTT, STT-2) were generally not associated among

each other in all dogs, except for a weak correlation between EAPPTT with PRT (r = 0.47; $P < 0.001$) and STT-2 (r = 0.20; $P = 0.0444$).

DISCUSSION

The present study investigated multiple diagnostic tools to measure aqueous tear production in a variety of canine breeds, repeating testing in each animal 24 hours later, thus providing valuable information that is currently sparse (or lacking) in the canine literature.

First, the study examined breeds of various cephalic conformations and showed significant differences in ocular surface diagnostics between brachycephalic and non-brachycephalic dogs. In particular, corneal sensitivity was significantly lower in brachycephalic *vs.* non-brachycephalic dogs, as determined by CTS (1.8 ± 0.5 cm *vs.* 3.4 ± 0.8 cm, respectively; $P < 0.001$), a finding likely related to anatomical differences in corneal innervation and subbasal nerve plexus.[15] Consequently, lacrimal tests that stimulate reflex tearing by touching the adnexa or ocular surface (*e.g.* STT, PRTT, EAPPTT) resulted in lower measurements in brachycephalic *vs.* non-brachycephalic dogs; in contrast, test results did not differ between cephalic conformations for SMT, a diagnostic tool that only touches the lacrimal lake and minimizes reflex tearing.[6] Clinically, these findings could help explain why the damage to corneal nerves from ocular surgery is often more detrimental in brachycephalic *vs.* non-brachycephalic dogs. Corneal sensitivity, reduced following phacoemulsification (due to corneal incision)[33] and transscleral cyclophotocoagulation (due to thermal injury),[25,34] may drop below a certain threshold in brachycephalic dogs and predispose the eye to develop ocular surface complications such as aqueous tear deficiency and neurotrophic keratopathy.[25,34,35] Such complications are presumably less common in non-brachycephalic dogs given their higher corneal sensitivity and aqueous tear production at baseline.

Second, the study showed that results of lacrimal tests were correlated for only selected pairwise comparisons, and the correlation strength was often weak to moderate (*r* between 0.20-0.59). For instance, a positive but moderate correlation was noted between STT-1 and STT-2 ($r = 0.39$) as well as STT-1 and PRTT ($r = 0.43$), as previously described in dogs,[3,6] cats[11] and humans.[36] The relatively poor correlations among lacrimal tests suggest that a comprehensive assessment of the ocular surface requires the combination of several diagnostic tools, as recently described in West Highland White Terriers affected with spontaneous aqueous deficient dry eye.[4] In fact, the combination of lacrimal tests can improve the clinician's diagnostic capability: in humans with dry eye disease, the combination of STT-1 and PRTT significantly improved the ability to diagnose ocular dryness, with optimized positive and negative predictive values compared to STT-1 alone,[37] and the same may be true in canine patients.

Third, another important finding from the present study was the relatively low test-retest repeatability for all ocular diagnostics assessed (ICC $\leq$ 0.75), except for corneal tactile sensation (ICC > 0.75). Similar findings have been reported in cats,[11] although the repeatability of lacrimal tests is generally better in the canine population with a higher ICC in dogs *vs.* cats for STT-1 (0.57 *vs.* 0.44) and PRTT (0.64 *vs.* 0.19). Of note, test-retest repeatability is generally improved in patients with diseased eyes,[10,38] thus the present findings should not be directly extrapolated to dogs with keratoconjunctivitis sicca or other ocular surface diseases. Further, it is important for clinicians to understand the difference between reliability and agreement, two distinct but complimentary aspects of test-retest repeatability.[39] When a test is repeated twice under similar conditions in a given population, reliability is defined as the capacity of the test to replicate the same ordering between subjects, while agreement represents the capacity of the test to provide strictly identical results in the same subjects.[39] Reliability, sometimes called test-retest correlation, indicates the degree of association between two sets of measurements (not their equality) and is thereby a necessary but not sufficient condition to demonstrate agreement.[39] Both aspects of test-retest

repeatability were assessed in the present study, using Pearson's correlation tests for reliability, and ICC and Bland-Altman plots for agreement. We showed that most diagnostics tests have a good reliability in dogs (except for STT-2 and EAPPTT in brachycephalic dogs), indicating that the assays can provide useful information for the comparison of different subjects. However, the agreement for ocular surface diagnostics was generally poor to moderate in dogs (except for corneal sensitivity), thus hindering the ability to distinguish between a real change in the examined patient (*i.e.* related to disease progression or therapeutic effects) and a random variation (*i.e.* measurement noise). The latter may be due to variations in the diagnostic test itself – for instance, changes in absorptive properties between Schirmer strip lots[40] – environmental factors (*e.g.* temperature, humidity), patient factors (*e.g.* anxiety) and examiner consistency.

The major neural pathway of lacrimation involves afferent input from the cornea and conjunctiva via the long ciliary nerves, a division of the ophthalmic branch of trigeminal nerve. When activated, this sensory input results in the stimulation of efferent parasympathetic and sympathetic nerves that promote tear secretion from the lacrimal gland and gland of the third eyelid (**Figure 4A**).[8] A secondary afferent pathway for lacrimation involves the activation of trigeminal afferent nerves in the nasal cavity, leading to an increase in activity in the superior salivatory nucleus, a region responsible for control of natural lacrimation (**Figure 4B**).[20] This neural pathway, termed nasolacrimal reflex (NLR), participates in basal tear secretion via stimulation of the nasal mucosa from nasal breathing – accounting for approximately one-third of basal tearing in humans[9] – and also plays an important role in responding to nasal foreign bodies or irritants by secreting tears into the nasal cavity via the nasolacrimal duct. NLR is well described in humans with reports as early as the 1950s[9,16-20,41] yet, to the authors knowledge, this is the first report of NLR in the canine species. In dogs, both the ethmoidal nerve (ophthalmic branch) and caudal nasal nerve (maxillary branch) participate to the innervation of the nasal mucosa.[42] In the present study, we

showed that sensory stimulation of the nasal mucosa significantly increased tear secretion by an average of 18% in all dogs, a finding that is likely to be more pronounced in dogs with keratoconjunctivitis sicca; indeed, a recent study in which NLR was utilized therapeutically in humans with dry eye disease reported an increase in lacrimation by 114-138% on average.[19] Here, the authors selected alcohol-soaked cotton balls for triggering NLR in dogs given the availability of these products in veterinary practice, yet other compounds such as ammonia may be used in future studies to provide a stronger sensory stimulation.[41] Similarly, the laterality of NLR was not specifically tested herein (as the cotton ball was placed in front of both nostrils) as studies in humans show that unilateral neurostimulation provides a bilateral lacrimal response (albeit stronger in the ipsilateral side).[9,17,18,43] In fact, a deeper understanding of NLR is much needed in dogs, especially given the relatively high prevalence of tear film deficiency in this species, and the numerous clinical and research implications. First, studying NLR could provide valuable insight into the interconnection between lacrimation and nasal cavity in dogs, both in healthy and disease state. For instance, it is plausible that the reduced/altered airflow in the nasal cavity of dogs with brachycephalic airway syndrome could explain the lower tear production seen in these canine breeds, or that dogs with rhinitis would have excessive tearing due to reflex aqueous secretion.[18] Moreover, the NLR could serve as an additional diagnostic tool for veterinary practitioners, for instance in dogs with corneal hypoesthesia secondary to diabetes mellitus[44] or transscleral cyclophotocoagulation;[25,34] indeed, the diagnostic utility of NLR was demonstrated in numerous human studies, allowing for differentiation between normal and dry eye subjects[41] or identification of cases with facial nerve palsy,[17] as well as a recent case report in a cat with neurogenic dry eye.[22] Last, the NLR represents a novel and potentially promising option for management of dry eye disease.[19,20] In particular, there is mounting evidence that nasal neurostimulation not only increases the aqueous component of tears but also the mucins,[45] proteins[46] and lipids,[47] thus providing ‘pure’ endogenous tears to relieve ocular symptoms of dryness.

The main limitation of the study is the low sample size (n = 50 dogs), partly due to the stringent inclusion criteria (*i.e.* only dogs with healthy ocular surface, for instance excluding any brachycephalic dog with discharge or keratitis). Although considered enough for testing test reliability,[48] the sample size is insufficient to establish proper 'reference values' for the various diagnostic tests examined herein.[49] In contrast, a recent study examined multiple lacrimal tests in a very large population (n = 621 dogs),[6] but the authors did not assess test-retest repeatability or the impact of cephalic conformation on the test values. Further, the experimental design only focused on diagnostic tests that measure the aqueous portion of the tears, and did not examine diagnostic tools associated with tear film quality (*e.g.* tear film breakup time, meibometry, tear ferning).

In summary, the present study shows the importance of corneal sensitivity (and closely related cephalic conformation) on the aqueous tear secretion in dogs, underlines the few correlations that exist among lacrimal tests, and highlights the generally good reliability but poor-to-moderate agreement when lacrimal tests are repeated twice under similar conditions in healthy canine subjects. Preliminary findings on NLR show that this neural pathway may represent a novel tool in canine ophthalmology, with potential diagnostic and therapeutic implications; although further characterization in healthy and diseased animals is first needed.

Conflict of interest statement

The authors declare no conflicts of interest.

ORCID

https://orcid.org/0000-0001-5093-6841

ACKNOWLEDGEMENTS

The authors are grateful to Alex Pereira, Maria Madalena Oliveira, Ana Paula da Silva and Professor Francisco de Assis Dórea Neto from the Federal University of Bahia for their technical assistance. APO received meniscometry strips for the clinical evaluations under-taken in this study and did not receive any grants, funding, or honorarium for the conduct of this research. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. APO is a research fellow from the National Council for Scientific and Technological Development (CNPq).

REFERENCES

1. Schirmer O. Studien zur Physiologie und Pathologie der Tränenabsonderung und Tränenabfuhr. *Albrecht von Graefes Archiv für Ophthalmologie* 1903;56:197-291.
2. Brown M, Galland J, Davidson H, et al. The phenol red thread tear test in dogs. *Veterinary and comparative ophthalmology (USA)* 1996.
3. Saito A, Kotani T. Estimation of lacrimal level and testing methods on normal beagles. *Vet Ophthalmol* 2001;4:7-11.
4. Leonard BC, Stewart KA, Shaw GC, et al. Comprehensive Clinical, Diagnostic, and Advanced Imaging Characterization of the Ocular Surface in Spontaneous Aqueous Deficient Dry Eye Disease in Dogs. *Cornea* 2019.
5. Lange RR, Lima L, Montiani-Ferreira F. Measurement of tear production in black-tufted marmosets (Callithrix penicillata) using three different methods: modified Schirmer's I, phenol red thread and standardized endodontic absorbent paper points. *Vet Ophthalmol* 2012;15:376-382.
6. Miyasaka K, Kazama Y, Iwashita H, et al. A novel strip meniscometry method for measuring aqueous tear volume in dogs: Clinical correlations with the Schirmer tear and phenol red thread tests. *Vet Ophthalmol* 2019.

7. Williams DL. Analysis of tear uptake by the Schirmer tear test strip in the canine eye. *Vet Ophthalmol* 2005;8:325-330.
8. Dartt DA. Neural regulation of lacrimal gland secretory processes: relevance in dry eye diseases. *Prog Retin Eye Res* 2009;28:155-177.
9. Gupta A, Heigle T, Pflugfelder SC. Nasolacrimal stimulation of aqueous tear production. *Cornea* 1997;16:645-648.
10. Nichols KK, Mitchell GL, Zadnik K. The repeatability of clinical measurements of dry eye. *Cornea* 2004;23:272-285.
11. Sebbag L, Kass PH, Maggs DJ. Reference values, intertest correlations, and test-retest repeatability of selected tear film tests in healthy cats. *J Am Vet Med Assoc* 2015;246:426-435.
12. Benz P, Tichy A, Nell B. Review of the measuring precision of the new Meibometer MB550 through repeated measurements in dogs. *Vet Ophthalmol* 2008;11:368-374.
13. Sebbag L, Park SA, Kass PH, et al. Assessment of tear film osmolarity using the TearLab(™) osmometer in normal dogs and dogs with keratoconjunctivitis sicca. *Vet Ophthalmol* 2017;20:357-364.
14. Maggio F. Ocular surface disease in dogs part 1: aetiopathogenesis and clinical signs. *Companion Animal* 2019;24:240-245.
15. Kafarnik C, Fritsche J, Reese S. Corneal innervation in mesocephalic and brachycephalic dogs and cats: assessment using in vivo confocal microscopy. *Vet Ophthalmol* 2008;11:363-367.
16. Boberg-Ans J. Experience in clinical examination of corneal sensitivity; corneal sensitivity and the naso-lacrimal reflex after retrobulbar anaesthesia. *Br J Ophthalmol* 1955;39:705-726.
17. Zilstorff-Pedersen K. Quantitative measurements of the nasolacrimal reflex. *Arch Otolaryngol* 1965;81:457-462.
18. Baroody FM, Foster KA, Markaryan A, et al. Nasal ocular reflexes and eye symptoms in patients with allergic rhinitis. *Ann Allergy Asthma Immunol* 2008;100:194-199.
19. Friedman NJ, Butron K, Robledo N, et al. A nonrandomized, open-label study to evaluate the effect of nasal stimulation on tear production in subjects with dry eye disease. *Clin Ophthalmol* 2016;10:795-804.
20. Sheppard JD, Torkildsen GL, Geffin JA, et al. Characterization of tear production in subjects with dry eye disease during intranasal tear neurostimulation: Results from two pivotal clinical trials. *Ocul Surf* 2019;17:142-150.

21. Pattar GR, Jerkins G, Evans DG, et al. Symptom improvement in dry eye subjects following intranasal tear neurostimulation: Results of two studies utilizing a controlled adverse environment. *The Ocular Surface* 2019.
22. Sebbag L, Pesavento PA, Carrasco SE, et al. Feline dry eye syndrome of presumed neurogenic origin: a case report. *JFMS Open Rep* 2018;4:2055116917746786.
23. Sebbag L, Allbaugh RA, Wehrman RF, et al. Fluorophotometric Assessment of Tear Volume and Turnover Rate in Healthy Dogs and Cats. *J Ocul Pharmacol Ther* 2019.
24. Evans HE, de Lahunta A. The Skeleton In: Evans HE,de Lahunta A, eds. *Miller's Anatomy of the Dog*. 4th ed. Philadelphia: Saunders, 2013.
25. Sebbag L, Crabtree EE, Sapienza JS, et al. Corneal hypoesthesia, aqueous tear deficiency, and neurotrophic keratopathy following micropulse transscleral cyclophotocoagulation in dogs. *Vet Ophthalmol* 2019.
26. Sebbag L, Moody LM, Allbaugh RA, et al. Nerve growth factor in dogs: Assessment of two immunoassays and selected ocular parameters following a nicergoline challenge per os. *Vet Ophthalmol* 2019.
27. Tagawa Y, Noda K, Ohguchi T, et al. Corneal hyperalgesia in patients with short tear film break-up time dry eye. *Ocul Surf* 2019;17:55-59.
28. Wieser B, Tichy A, Nell B. Correlation between corneal sensitivity and quantity of reflex tearing in cows, horses, goats, sheep, dogs, cats, rabbits, and guinea pigs. *Vet Ophthalmol* 2013;16:251-262.
29. McAlinden C, Khadka J, Pesudovs K. Statistical methods for conducting agreement (comparison of clinical tests) and precision (repeatability or reproducibility) studies in optometry and ophthalmology. *Ophthalmic Physiol Opt* 2011;31:330-338.
30. Campbell MJ, Swinscow TDV. Correlation and regression. *Statistics at square one*: Wiley-Blackwell Chichester, UK, 2009.
31. Bland JM, Altman DG. Measuring agreement in method comparison studies. *Stat Methods Med Res* 1999;8:135-160.
32. Portney LG, Watkins MP. *Foundations of clinical research: applications to practice*: Pearson/Prentice Hall Upper Saddle River, NJ, 2009.
33. Khanal S, Tomlinson A, Esakowitz L, et al. Changes in corneal sensitivity and tear physiology after phacoemulsification. *Ophthalmic Physiol Opt* 2008;28:127-134.

34. Sebbag L, Allbaugh RA, Strauss RA, et al. MicroPulse^{TM} transscleral cyclophotocoagulation in the treatment of canine glaucoma: Preliminary results (12 dogs). *Vet Ophthalmol* 2018.
35. Gemensky-Metzler AJ, Sheahan JE, Rajala-Schultz PJ, et al. Retrospective study of the prevalence of keratoconjunctivitis sicca in diabetic and nondiabetic dogs after phacoemulsification. *Vet Ophthalmol* 2015;18:472-480.
36. Senchyna M, Wax MB. Quantitative assessment of tear production: A review of methods and utility in dry eye drug discovery. *J Ocul Biol Dis Infor* 2008;1:1-6.
37. de Monchy I, Gendron G, Miceli C, et al. Combination of the Schirmer I and phenol red thread tests as a rescue strategy for diagnosis of ocular dryness associated with Sjogren's syndrome. *Invest Ophthalmol Vis Sci* 2011;52:5167-5173.
38. Nichols KK, Zadnik K. The repeatability of diagnostic tests and surveys in dry eye. *Adv Exp Med Biol* 2002;506:1171-1175.
39. Berchtold A. Test–retest: Agreement or reliability? *Methodological Innovations* 2016;9:2059799116672875.
40. Hawkins EC, Murphy CJ. Inconsistencies in the absorptive capacities of Schirmer tear test strips. *J Am Vet Med Assoc* 1986;188:511-513.
41. Maffei CJ, Iii GWO, Welch DL, et al. Time to Reflex Tearing in Response to Chemical Nasolacrimal Stimulus in Dry Eye Subjects versus Normals. *Investigative Ophthalmology & Visual Science* 2010;51:3398-3398.
42. Evans HE, De Lahunta A. Cranial nerves. *Miller's anatomy of the dog 4th edn St Louis: Elsevier-Saunders* 2013:708-729.
43. Drummond PD. Lacrimation and cutaneous vasodilatation in the face induced by painful stimulation of the nasal ala and upper lip. *J Auton Nerv Syst* 1995;51:109-116.
44. Good KL, Maggs DJ, Hollingsworth SR, et al. Corneal sensitivity in dogs with diabetes mellitus. *Am J Vet Res* 2003;64:7-11.
45. Gumus K, Schuetzle KL, Pflugfelder SC. Randomized Controlled Crossover Trial Comparing the Impact of Sham or Intranasal Tear Neurostimulation on Conjunctival Goblet Cell Degranulation. *Am J Ophthalmol* 2017;177:159-168.
46. Woodward A, Senchyna M, Franke M, et al. Effect of Intranasal Neurostimulation on Tear Protein Content in Patients With Dry Eye. *Investigative Ophthalmology & Visual Science* 2017;58:2673-2673.

47. Green KB, Kamat M, Franke M, et al. Tear Total Lipid Concentration in Patients with Dry Eye Following Intranasal Neurostimulation. *Investigative Ophthalmology & Visual Science* 2017;58:2693-2693.
48. Springate SD. The effect of sample size and bias on the reliability of estimates of error: a comparative study of Dahlberg's formula. *Eur J Orthod* 2012;34:158-163.
49. Horowitz GL. Reference Intervals: Practical Aspects. *EJIFCC* 2008;19:95-105.

TABLES

Table 1. Mean ± standard deviation (range) values of ocular surface diagnostics in 50 dogs, including 25 brachycephalic dogs and 25 non-brachycephalic dogs.

	All (*n* = 50)	**Brachycephalic** (*n* = 25)	**Non-brachycephalic** (*n* = 25)
CTS (cm)	2.6 ± 1.1 (1.0-5.0)	1.8 ± 0.5 (1.0-3.5)	3.4 ± 0.8 * (2.0-5.0)
CTS (g/mm^2)	2.9 ± 2.7 (0.55-10.3)	4.7 ± 2.7 (1.0-10.3)	1.3 ± 0.6 * (0.55-2.8)
SMT (mm/5s)	7.3 ± 2.2 (2.0-14.0)	7.4 ± 2.0 (2.0-11.0)	7.3 ± 2.4 (3.0-14.0)
PRTT (mm/15s)	39.2 ± 5.1 (27.0 50.0)	37.2 ± 4.0 (27.0-46.0)	41.1 ± 5.5 * (30.0-50.0)
EAPPTT (mm/15s)	17.1 ± 2.8 (11.0-25.0)	16.6 ± 2.7 (11.0-22.0)	17.5 ± 2.9 (11.0-25.0)
STT-1 (mm/min)	21.7 ± 4.9 (12.0-35.0)	20.1 ± 3.4 (12.0-26.0)	23.3 ± 5.7 * (14.0-35.0)
NL-STT1 (mm/min)	25.1 ± 4.9 (14.0-35.0)	23.2 ± 3.6 (14.0-31.0)	27.1 ± 5.4 * (17.0-35.0)
STT-2 (mm/min)	14.9 ± 4.1 (7.0-29.0)	13.0 ± 3.4 (7.0-22.0)	16.9 ± 3.9 * (10.0-29.0)

An asterisk (*) indicates statistical difference ($P < 0.05$) between brachycephalic and non-brachycephalic dogs. CTS = Corneal tactile sensation; SMT = Strip meniscometry test; PRTT = Phenol red thread test; EAPPTT = Endodontic absorbent paper point tear test; STT-1 = Schirmer tear test-1; NL-STT1 = Schirmer tear test-1 with nasolacrimal stimulation; STT-2 = Schirmer tear test-2.

Table 2. Test-retest repeatability of ocular surface diagnostics in 50 dogs, including 25 brachycephalic dogs and 25 non-brachycephalic dogs, evaluating the reliability (light gray boxes) and agreement (dark gray boxes) for each diagnostic test. See Table 1 for the detail of the abbreviations used.

	RELIABILITY			**AGREEMENT**		
	Pearson's coefficient r (*P* value)			Intraclass correlation coefficient (95% limits of agreement)		
	All (*n* = 50)	Brachycephalic (*n* = 25)	Non-brachycephalic (*n* = 25)	All (*n* = 50)	Brachycephalic (*n* = 25)	Non-brachycephalic (*n* = 25)
CTS (cm)	0.92 (*P* < 0.001)	0.79 (*P* < 0.001)	0.79 (*P* < 0.001)	0.91 (-1.0 to 0.8)	0.78 (-0.8 to 0.7)	0.77 (-1.2 to 0.8)
SMT (mm/5s)	0.56 (*P* < 0.001)	0.62 (*P* < 0.001)	0.60 (*P* = 0.001)	0.57 (-4.1 to 3.9)	0.57 (-3.5 to 4.0)	0.58 (-4.7 to 3.8)
PRTT (mm/15s)	0.64 (*P* < 0.001)	0.51 (*P* = 0.009)	0.67 (*P* < 0.001)	0.64 (-8.9 to 8.3)	0.49 (-6.6 to 8.9)	0.65 (-10.3 to 7.0)
EAPPTT (mm/15s)	0.24 (*P* = 0.010)	- 0.04 (*P* = 0.853)	0.47 (*P* = 0.020)	0.24 (-7.0 to 6.7)	0.04 (-7.2 to 8.1)	0.47 (-6.6 to 5.1)
STT-1 (mm/min)	0.59 (*P* < 0.001)	0.57 (*P* = 0.003)	0.56 (*P* = 0.004)	0.57 (-9.2 to 8.9)	0.54 (-7.4 to 4.8)	0.53 (-10.0 to 11.9)
NL-STT1 (mm/min)	0.72 (*P* < 0.001)	0.69 (*P* < 0.001)	0.70 (*P* < 0.001)	0.73 (-7.0 to 7.4)	0.65 (-6.2 to 5.8)	0.69 (-7.7 to 8.9)
STT-2 (mm/min)	0.58 (*P* < 0.001)	0.21 (*P* = 0.312)	0.65 (*P* < 0.001)	0.56 (-7.9 to 7.5)	0.21 (-8.4 to 8.4)	0.59 (-7.5 to 6.6)

Table 3. Pearson's correlation coefficients (r) describing the association between ocular surface diagnostics in dogs, detailing results in all dogs (n = 50), brachycephalic dogs (n = 25) and non-brachycephalic dogs (n = 25). A statistically significant correlation is denoted by an asterisk (* for $P < 0.05$, and ** for $P < 0.01$).

		STT-2 (mm/min)	**NL-STT1** (mm/min)	**STT-1** (mm/min)	**EAPPTT** (mm/15s)	**PRTT** (mm/15s)	**SMT** (mm/5s)
CTS (cm)	All	0.21 *	0.10	0.02	0.13	0.28 **	0.15
	Brachycephalic	-0.22	-0.25	-0.32 *	-0.08	-0.25	-0.02
	Non-brachycephalic	-0.33 *	-0.40 **	-0.42 **	0.08	0.01	0.44 **
SMT (mm/5s)	All	-0.18	-0.05	-0.05	0.16	0.09	
	Brachycephalic	0.01	-0.05	-0.05	0.14	0.03	
	Non-brachycephalic	-0.34 *	-0.04	-0.05	0.18	0.16	
PRTT (mm/15s)	All	0.13	0.51 **	0.43 **	0.47 **		
	Brachycephalic	-0.14	0.50 **	0.45 **	0.26		
	Non-brachycephalic	-0.01	0.39 **	0.30 *	0.58 **		
EAPPTT (mm/15s)	All	0.20 *	0.15	0.19			
	Brachycephalic	0.16	0.02	0.14			
	Non-brachycephalic	0.14	0.15	0.16			
STT-1 (mm/min)	All	0.39 **	0.83 **				
	Brachycephalic	0.11	0.83 **				
	Non-brachycephalic	0.38 **	0.80 **				
NL-STT1 (mm/min)	All	0.38 **					
	Brachycephalic	-0.16					
	Non-brachycephalic	0.47 **					

FIGURES

Figure 1. Representative photographs demonstrating the following ocular surface diagnostics in dogs: Corneal esthesiometry (A), strip meniscometry test (B), phenol red thread test (C), endodontic absorbent paper point tear test (D), Schirmer tear test-1 (E) and Schirmer tear test-1 with nasolacrimal stimulation (F).

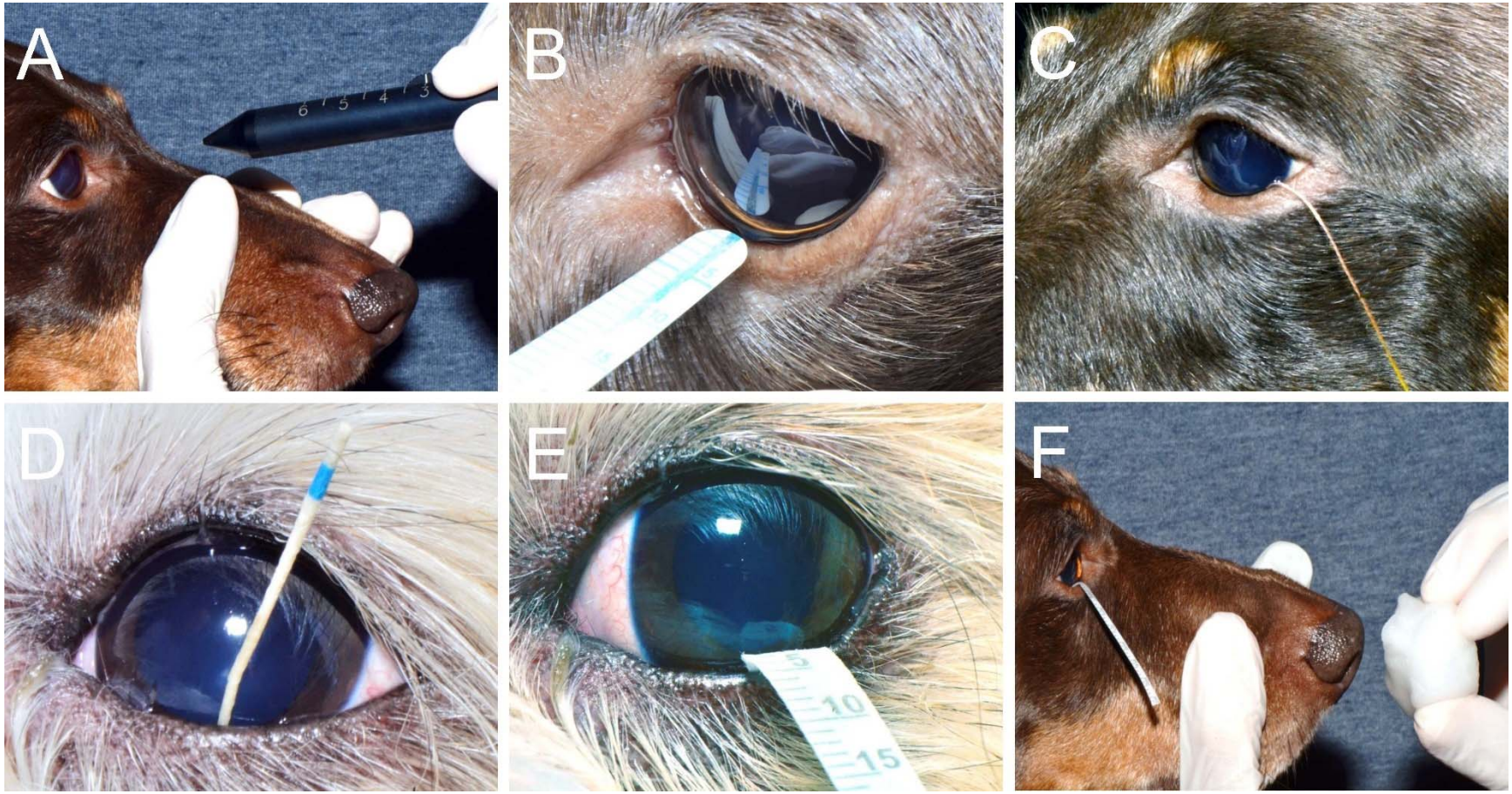

Figure 2. Bland-Altman plots displaying test-retest repeatability for the corneal tactile sensation (A), strip meniscometry test (B), phenol red thread test (C), endodontic absorbent paper point tear test (D), Schirmer tear test-1 (E), Schirmer tear test-1 with nasal neurostimulation (F), and Schirmer tear test-2 (G).The vertical axis represents the difference between repeated measurements, and the horizontal axis plots the mean value for the 2 sessions. The mean of the differences is represented by the middle horizontal line intersecting the vertical axis and should be close to zero. The lower and upper horizontal lines represent the 95% limits of agreement.

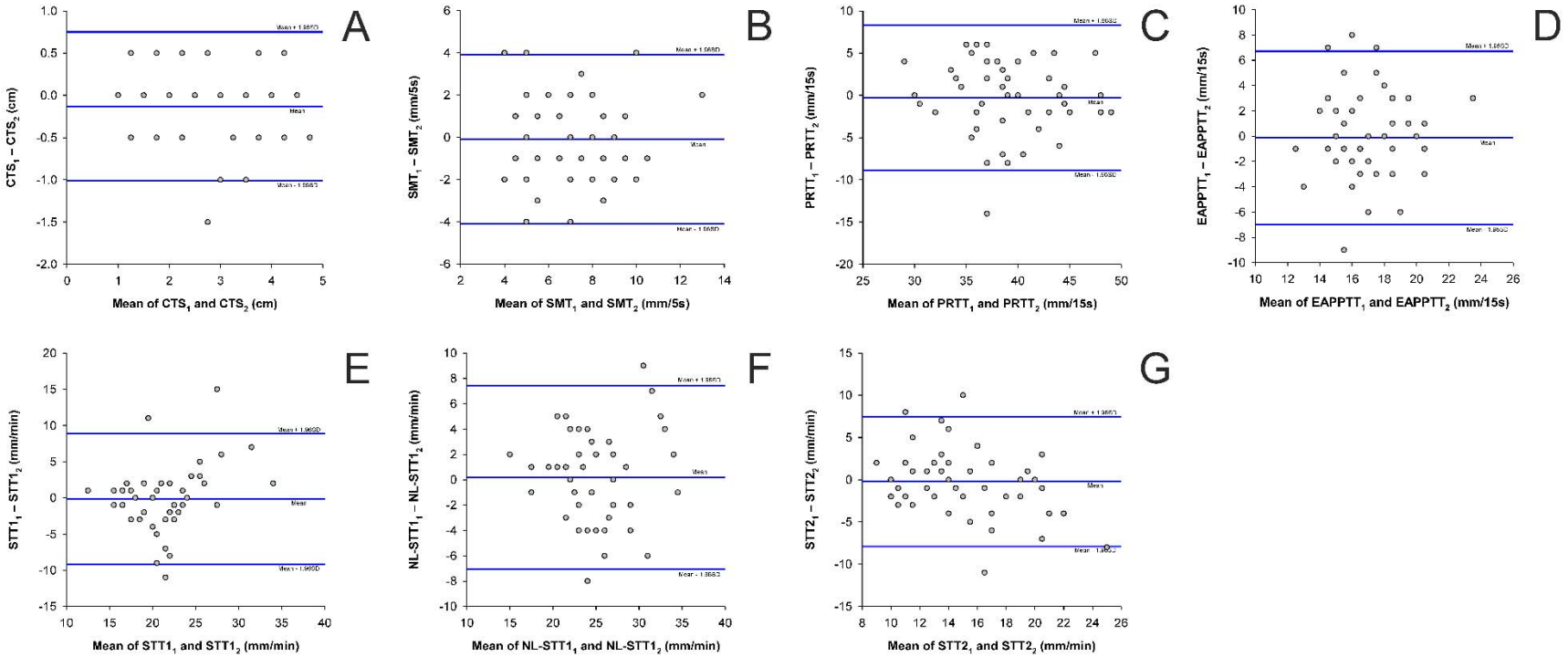

Figure 3. Results of the Pearson correlation testing between the cephalic index and the following ocular surface diagnostics: corneal tactile sensation (A), phenol red thread test (B), Schirmer tear test-1 with nasal neurostimulation (C), and Schirmer tear test-2 (D).

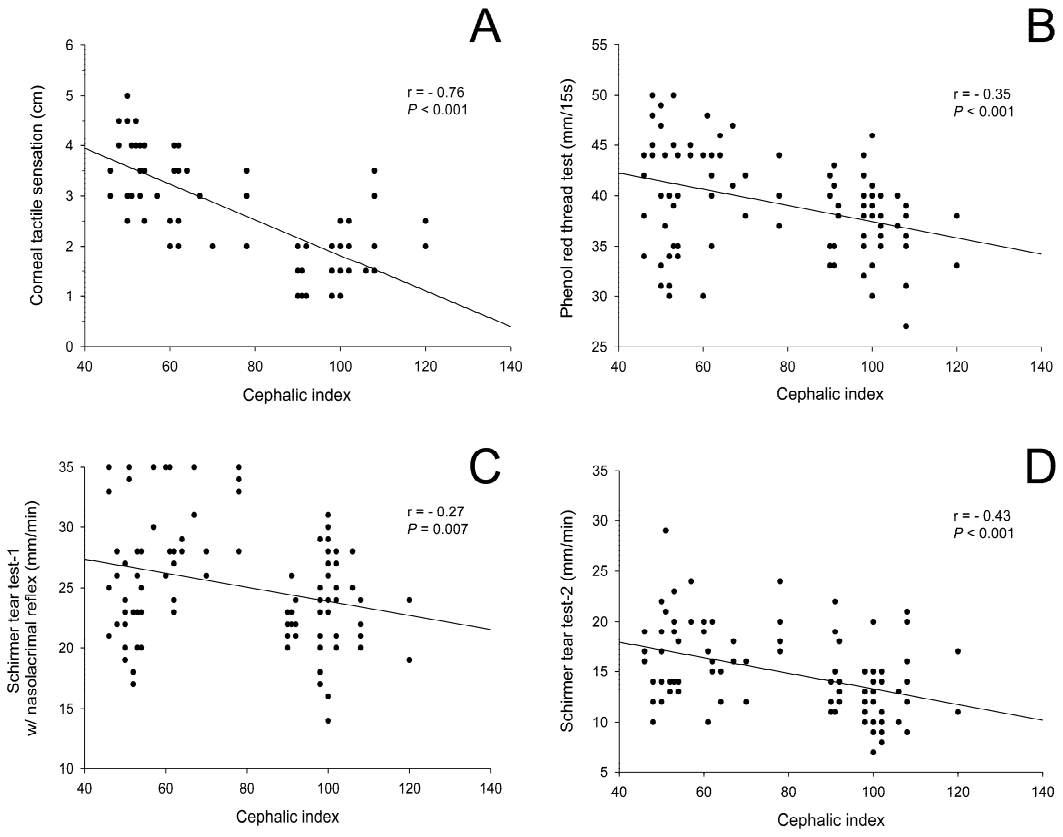

Figure 4. (A) Lacrimation reflex following standard Schirmer testing, involving afferent input from the ocular surface via the long ciliary nerves (ophthalmic branch of trigeminal nerve) that result in efferent stimulation of tear secretion from the lacrimal gland and gland of the third eyelid. (B) Lacrimation reflex following Schirmer testing concurrently to nasal neurostimulation with an alcohol-soaked cotton ball (nasolacrimal reflex), involving afferent input from the ocular surface and the nasal mucosa (ethmoidal nerve of ophthalmic branch and caudal nasal nerve of maxillary branch) and a similar efferent pathway.