Graham Kathleen (Orcid ID: 0000-0003-2548-2969)

**Protocol for assessment of the pupillary light reflex in dogs without chemical restraint:
preliminary investigation**

Kathleen L. Graham^a BVSc(Hons1) MVS MANZCVS PhD

Christina I McCowan^{b,c} BVSc BSc(Hons) PhD MANZCVSc DACVP

Andrew White^{a,d} BMedSci(Hons) MBBS PhD FRANZCO

^a Clinical Ophthalmology and Eye Health, Sydney Medical School, University of Sydney, NSW
Australia

^b University of Melbourne Veterinary Hospital, University of Melbourne; Victoria Australia

^c Agriculture Victoria, Bundoora Australia 3083

^{a,d} Westmead Institute for Medical Research, Westmead, NSW Australia 2145

Corresponding author: Kathleen Graham

Email: kathleen.graham@sydney.edu.au

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Kathleen L. Graham^a BVSc(Hons1) MVS MANZCVS PhD

Christina I McCowan^{b,c} BVSc BSc(Hons) PhD MANZCVSc DACVP

Andrew White^{a,d} BMedSci(Hons) MBBS PhD FRANZCO

^a Clinical Ophthalmology and Eye Health, Sydney Medical School, University of Sydney,
NSW Australia

^b University of Melbourne Veterinary Hospital, University of Melbourne; Victoria Australia

^c Agriculture Victoria, Bundoora Australia 3083

^{a,d} Westmead Institute for Medical Research, Westmead, NSW Australia 2145

Corresponding author: Kathleen Graham

Email: kathleen.graham@sydney.edu.au

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Objective: To explore the use of a modified pupillometry technique in dogs without chemical restraint

Materials and Methods: Following dark adaptation, pupillary light reflexes were assessed in six dogs with sudden acquired retinal degeneration syndrome (SARDS), the unaffected eye of eight dogs with unilateral primary glaucoma ('predisposed'), and in 11 healthy dogs. Responses to red, blue and white lights were recorded and relative pupil sizes subsequently determined based on video recordings of each test.

Results: Mean testing time was 2.3 minutes, excluding time for dark adaptation. Baseline pupil size in dogs with SARDS was greater than in normal and predisposed eyes. Pupil constriction was reduced in predisposed compared to normal and SARDS eyes when stimulated with high intensity blue light. Compared to normal eyes, those with SARDS had reduced pupil constriction when stimulated with low and high intensity red light, low intensity blue light and white light.

Clinical Importance: Quantitative measures of pupil function were obtained from healthy and diseased eyes without the need for chemical restraint. Further investigations are warranted to validate the technique and evaluate its use in the management of canine glaucoma.

Introduction

The pupillary light reflex (PLR) is an autonomic reflex controlling pupil diameter in response to light. For a long time, the PLR was believed to rely only on the activation of rod and cone photoreceptors in dim and daylight, respectively. (Loewenfeld, 1993) However, measurable PLR activity was identified in the absence of rods and cones (Lucas *et al.* 2001) and melanopsin (Hattar *et al.* 2002) was found to cause a light response in intrinsically photosensitive retinal ganglion cells (ipRGCs) (Berson *et al.* 2002). Based on these findings, we now know that the PLR is driven by rod-, cone- and ganglion cell-mediated activity. As the spectral sensitivity of ipRGCs differs from that of rod and cone photoreceptors, assessment of pupil responses to different wavelengths of light permits evaluation of rods, cones and ipRGCs. Pupillometry is a non-invasive, objective method that provides measures of pupil size and response. With the physiological differences between cellular responses within the retina, chromatic pupillometry - measuring pupil responses to different wavelengths and intensities of light - becomes a useful tool in evaluating visual function and has been used in assessment of glaucoma in people (Adhikari *et al.* 2016, Feigl *et al.* 2011, Kankipati *et al.* 2011, Nissen *et al.* 2014) and pupillometry techniques are also described in dogs (Grozdanic *et al.* 2013, Grozdanic *et al.* 2019, Grozdanic *et al.* 2007, Kim *et al.* 2015, Whiting *et al.* 2013a, Whiting *et al.* 2015, Whiting *et al.* 2013b).

Chromatic pupillometry has been reported in investigations of the pathophysiology of sudden acquired retinal degeneration syndrome (SARDS). With the need for concerted efforts to improve our ability to detect and manage canine glaucoma (Komáromy *et al.* 2019), investigation of the potential value of chromatic PLR assessments in glaucoma is prudent. Monitoring of pupil responses in canine glaucoma is complicated by changes

associated with the disease such as the use of topical prostaglandin-analogues that cause miosis, the development of peripheral anterior synechiae associated with disease, and iris ischaemia resulting from elevated intraocular pressure (IOP) (Miller *et al.* 2015). These factors limit the use of pupillometry in glaucomatous eyes but, if neuroretinal function is impaired before the onset of these changes, chromatic pupillometry may have potential use in the identification of early changes associated with the canine glaucomas.

To our knowledge, there are no published reports describing quantitative measures of pupil function in dogs without chemical restraint. In this study, we explore a technique to determine whether quantitative assessment of chromatic PLRs in fully conscious dogs was feasible in a veterinary clinical setting. Our secondary aim was to investigate whether there were differences in chromatic PLRs in eyes predisposed to glaucoma, and eyes with and without established disease.

Materials and Methods

Animals

Twenty-five pet dogs registered as patients in New South Wales veterinary clinics were included. A complete ophthalmic examination, including slit lamp biomicroscopy (Keeler PSL Classic Portable Slit Lamp, Keeler Ltd, UK), indirect ophthalmoscopy (Welch Allyn Australia Pty Ltd, NSW Australia), quantitative tear testing (Schirmer tear test I, Merck Animal Health, NJ, USA), rebound tonometry (Icare® Tonovet, Icare, Finland) and fluorescein staining, was performed in all cases. Medical records were reviewed and signalment, ophthalmic findings and treatments, concurrent disease and treatments, were recorded for analysis.

Based on clinical examination and history, dogs were allocated into groups: (i) '*predisposed*': eyes with an abnormal drainage angle on gonioscopy (narrowed iridocorneal angle and/or pectinate ligament dysplasia). For inclusion in this group, the contralateral eye had to have been diagnosed with primary glaucoma (clinical and/or histopathological) by a veterinary ophthalmologist; (ii) *SARDS*: eyes of dogs that presented to a veterinary ophthalmologist for sudden onset of bilateral blindness, with a diagnosis of *SARDS* made based on the absence of electrical activity on ERG and any visible abnormalities on ophthalmoscopy. Healthy dogs without evidence of clinical or historical ophthalmic or systemic disease were selected as controls.

When dogs had two eyes suitable for testing according to study criteria (healthy controls and dogs with *SARDS*) only the left eye was included in analyses of pupil responses. This was to avoid any potential impact of indirect stimulation of the contralateral eye on pupil measurements and to avoid bias from inclusion of two eyes from some (but not all) dogs.

Procedures

All procedures were conducted with approval from the University of Sydney Animal Ethics Committee (2016/1004), and with each dog owner's consent. PLRs were assessed in a darkened room after 10 minutes of dark adaptation. Each session was recorded using an infra-red video camera set up (1/2" 4-12mm F1.4 aspherical manual CMount infra-red lens, Tamron, Saitama Japan; CMOS, 87.2 fps, 752 x 480, 0.36 MPixel, 1/3", Global shutter, Aptina Imaging Corp. San Jose, California US). The left and right eyes were tested in each case, except in predisposed dogs in which only the predisposed eye was assessed. To avoid influence of indirect stimulation of the pupil in the second eye fitting study criteria (control and *SARDS* eyes), only measurements of the first eye

tested were used in analyses. The eyelids were held open using an eyelid speculum if tolerated by the dog, or the investigator's hands if the eyelid speculum was not tolerated, or did not allow appropriate imaging of the pupil responses. The BPI-50 Precision Illuminator (Retinographics, Inc., CT, USA) was used to stimulate pupillary function under low (1 000 lux +/- 5%) and high intensity (10 000 lux +/- 5%) red (660nm) and blue light (465nm) when held 4 cm from the dog's eye. The response to red light was assessed with 5 seconds of low intensity stimulus, then 5 seconds with high intensity light stimulation. After 10 minutes dark adaptation to allow pupil recovery, the PLR was assessed using the same protocol with a blue light. Assessment of PLR under white light conditions was performed using a slit lamp after a further 10 minutes dark adaptation. For each dog, this procedure was repeated in its entirety with a minimum of 2 hours interval, and the average pupil size from both sessions was used for analyses. A proportion of dogs returning for reassessment underwent a repeat of this testing procedure to determine test-retest reliability. Repeat testing was performed within 7 days when the dog had been diagnosed with a disease that could affect pupil function.

All video recordings were reviewed by the same investigator to determine the point of maximum constriction for each light stimulus. Still images from the video recording were obtained for analysis based on the following criteria: (1) the head and globe were positioned so that the iris plane was parallel to the camera, (2) a minimum of 180° of continuous corneal circumference was visible, and (3) the circumference of the pupil was clearly demarcated (Fig.1). Using open source ImageJ software (WS, R. 1997-2018) the pupil size was measured as a ratio of the corneal diameter at the following time points: resting pupil (after dark adaptation and before stimulation with any light source), and at the point of maximum miosis when stimulated with low intensity red light, high intensity red light, low intensity blue light, high intensity blue light, and white light. Relative pupil

constriction was then calculated using the following formula: (relative pupil diameter at maximum constriction) / (relative resting pupil diameter). Results of the relative pupil size are reported as 'pupil size' (Fig.2).

Statistical analyses

Statistical analyses were performed using commercially available software (GraphPad Prism 7). Kruskal-Wallis tests were used to compare the relative degree of pupil constriction between groups. Dunn's multiple comparisons test was then performed to further assess rank differences between groups for which a statistically significant difference was detected. Test-retest reliability was assessed using the intraclass correlation coefficient (ICC). A one-way random model was assumed where the subjects (dogs) are assumed random. This correlation was interpreted as excellent if >0.90, good when 0.80-0.89, adequate when 0.70-0.79 and with limited applicability if <0.70. Spearman's correlation coefficient was used to measure the strength of associations between relative pupil sizes and baseline characteristics including age and IOP. To identify a possible correlation between the degree of pupil constriction with each light stimulus and the duration of disease, predisposed eyes were categorised as acute (<1 week) or chronic (>6 months) based on the time since signs consistent with glaucoma had been noted in the contralateral eye. The degree of pupil constriction for acute *versus* chronic eyes was compared using a Mann-Whitney test. For eyes with SARDS, the duration of disease was categorised as acute (<1 week), short term (2 months – 1 year) and chronic (>1 year), and a Kruskal-Wallis test used to compare groups. For all these exploratory analyses, a *p* value <0.05 was considered statistically significant.

Results

Twenty-five dogs (25 eyes) were included. Eight dogs were predisposed to glaucoma and six dogs were diagnosed with SARDS. Eleven dogs served as the control population. The age, sex, proportion of purebred dogs, and IOP were similar between groups (Table 1). There was not a statistically significant correlation between age and the degree of pupil constriction in this study (overall, nor in individual groups).

Twenty-four of the 25 dogs enrolled in the study tolerated complete assessment of PLRs under red, blue and white light. Recording of the PLR in response to white light was not tolerated in one predisposed dog, although all other recordings were suitable for inclusion. Two assessments (one eye in two different dogs) were excluded due to positioning that precluded appropriate pupil measurements (Fig.3). Excluding time for dark adaptation (total 30mins), the average time to record PLRs in both eyes was 2.3 minutes (1.8-3.1 minutes).

Baseline pupil size

Baseline relative pupil size in dogs with SARDS (0.89 of the corneal diameter; range 0.87-0.89) was greater compared to both normal (0.80; range 0.76- 0.85; $p=0.002$) and predisposed eyes (0.80; range 0.78- 0.83; $p=0.002$). There was not a statistically significant difference in baseline pupil size between normal and predisposed eyes ($p>0.999$).

Red light stimulation

Eyes with SARDS had a greater relative pupil size (low intensity: 1.00, range 0.99-1.03; high intensity: 0.99, range 0.95-1.04) compared to both normal (low: 0.73, range 0.54-0.99; high: 0.54, range 0.39-0.70) and predisposed eyes (low: 0.75, range 0.66-0.87; high: 0.67, range 0.50-0.79; when stimulated by both low (*versus* normal $p=0.004$;

versus predisposed $p=0.013$) and high intensity red light (versus normal $p<0.001$; versus predisposed $p=0.045$) (Table 2) (Fig.4). There was not a statistically significant difference in relative pupil size between normal and predisposed eyes when stimulated with low ($p>0.999$) or high intensity ($p=0.322$) red light stimulation (Table 2).

Blue light stimulation

When stimulated with low intensity blue light, relative pupil size of SARDS eyes (median 0.71; range 0.52-0.82) was greater compared to normal eyes (0.49; range 0.39-0.70; $p=0.031$), but did not differ from predisposed eyes (0.61; range 0.56-0.95; $p>0.999$) (Fig.5). There was not a statistically significant difference in relative pupil size of normal and predisposed eyes ($p=0.105$).

When stimulated with high intensity blue light, relative pupil size of predisposed eyes (0.52; range 0.47-0.65) was greater than both normal (0.39; range 0.33-0.57; $p=0.002$) and SARDS eyes (0.40; range 0.36-0.45; $p=0.036$) (Fig.5) (Table 2). There was not a statistically significant difference in relative size of normal and SARDS pupils when stimulated with high intensity blue light ($p>0.999$).

White light stimulation

The relative size of SARDS pupils (0.80; range 0.77-0.88) was greater than pupils in normal (0.54; range 0.37-0.83; $p=0.007$) and predisposed eyes (0.55; range 0.46-0.66; $p=0.018$) (Fig.6) (Table 2). There was not a statistically significant difference in relative size of normal and predisposed pupils when stimulated with white light ($p>0.999$).

Test-retest reliability

Six dogs (six eyes) predisposed to glaucoma had the same protocol for assessment of colorimetric PLRs repeated between 3-15 days (median 6 days) after the initial assessment. Ophthalmic examination in all cases revealed no clinical evidence of progressive disease. There was excellent test-retest reliability in the degree of pupil constriction achieved with red, blue and white light stimulation (ICC 0.915).

Discussion

In this study, we provide preliminary data demonstrating that quantitative measures of pupil function in dogs can be determined without sedation or anaesthesia, using readily available equipment. In doing so, we identified a measurable reduction in pupil response to white and red light stimuli in eyes with SARDS, and decreased pupillary response in eyes predisposed to primary angle-closure glaucoma in response to high intensity blue light stimuli when compared other groups in this study. Pupil responses in SARDS cases were consistent with existing reports (Grozdanic *et al.* 2019, Grozdanic *et al.* 2007, Komáromy *et al.* 2016, Young *et al.* 2018) and other retinal diseases (Yeh *et al.* 2017), providing a form of external validation for our protocol. We suggest quantifying pupil responses with technology that is affordable to veterinarians may facilitate better assessment and monitoring of neuroretinal function in veterinary practice.

We found reduced pupil responses in predisposed compared to normal eyes when stimulated by high, but not low intensity blue light. Extrapolating from findings described by Yeh *et al.* (2017), these findings could reflect similarities in rod function between normal and predisposed eyes, with poorer ipRGC function in predisposed eyes accounting for the altered response to high intensity blue light. Although low intensity blue light resulted in reduced pupil responses in SARDS compared to normal eyes, high

intensity blue light elicited similar pupil responses in normal and SARDS eyes. Rod-driven pupil responses are purportedly mediated through ipRGCs (Güler *et al.* 2008), and attenuated pupil responses irrespective of the stimulus wavelength are reported in people with early primary open-angle glaucoma, suggesting altered synaptic transmission to ipRGCs, or reduced responsiveness of the outer photoreceptors in these eyes (Najjar *et al.* 2018). We suggest that the reduced pupil responses to high intensity blue light in eyes predisposed to glaucoma that we detected may indicate ipRGC signalling is affected.

Studies using formal pupillometry protocols that require chemical restraint describe an absent PLR with high intensity red light stimulation, and complete pupil constriction following high intensity blue light stimulation in all dogs with SARDS (Grozdanic *et al.* 2019, Grozdanic *et al.* 2007). We did not replicate these findings but instead, our results are similar to those reported in another study that did not use chemical restraint during pupillometry (Terakado *et al.* 2013). Those investigators suggested variation in their cases (dogs with progressive retinal atrophy) from existing reports may be attributed to progressive degeneration of photoreceptor and ganglion cells, and we speculate that dogs with SARDS in our study might also be similarly affected. Our study included dogs both acutely and chronically affected by SARDS, whereas previous reports include only dogs more acutely affected (Grozdanic *et al.* 2019, Young *et al.* 2018).

There are important limitations in interpreting these results. In attempting to quantify pupil responses using technology commonly available in clinical settings and avoiding the risks and costs associated with sedation and/or anaesthesia, our study fails to replicate some conditions used in formal pupillometry assessments. These conditions include the head/eye stability (with chemical restraint), the use of dogs of the same age and

duration of disease, and the use of background lighting to accurately assess different photosensitive retinal cell populations. Further studies necessary to validate this technique should investigate how pupil responses correlate with the number of optic nerve axons and whether age-related changes to the iris sphincter muscle have any impact on findings if there is no evidence of iris atrophy on ophthalmoscopy. Until physiological studies assessing such parameters are conducted with suitably powered studies in healthy and diseased animals across heterogeneous populations, the mechanisms by which responses are altered cannot be determined. Although our protocol measured relative pupil constriction as the outcome as a means to compare between groups of dogs in which pupil size is affected by disease, we cannot rule out potential influences on baseline pupil size, such as disease status, stress, and age that could affect results. However, investigating techniques that could allow for quantitative assessment of large populations is important in developing our understanding of disease, and to facilitate appropriate management of clinical cases.

Conducting pupillometry in conscious dogs is advantageous in that the variability in pupil responses associated with different drugs was avoided (Gunderson *et al.* 2013, Stephan *et al.* 2003). However, problems including head and eye movement and blinking are introduced. Despite this, only one dog did not tolerate the full protocol, and all dogs tolerated complete assessments with red and blue light. Relatively short periods of testing (1.8-3.1 minutes) were broken up by 10 minute 'breaks' for dark adaptation between stimuli, meaning procedural timing for the dogs did not appear to cause undue stress. With the addition of a total 30 minutes dark adaptation (in 10 minute intervals), this testing procedure does take a considerable amount of time, although actual testing is not prolonged and was well tolerated by the dogs. By attempting to develop a protocol practical in routine veterinary assessments, conditions in this study were not optimised

for specific and isolated testing of individual photoreceptor layers and ipRGCs as they have been in previous reports describing optimal conditions for these assessments (Yeh *et al.* 2017). For example, the low intensity blue light in our protocol was the equivalent of 1.6cd which is higher than that described (1cd/m²) (Yeh *et al.* 2017) to specifically assess rod-mediated responses. Despite these limitations, our results show there is merit in further investigations using our modified protocol. We believe there are merits in investigating a protocol that would allow assessment of large numbers of dogs in a heterogenous population, and this is not practical with previously described formal protocols. (Grozdanic *et al.* 2007, Yeh *et al.* 2017)

The sample size in our study was small due to the clinical nature of this prospective preliminary study. Although we investigated correlations between variables in each of the groups, the small sample size, and the inability to definitively account for the chronicity of disease in each group limits the information we can obtain about each disease. Furthermore, there is a clear degree of 'overlap' in the quantitative measures obtained in this study, precluding the use of such parameters as a stand-alone diagnostic test. Validation with concurrent ERG studies, a larger sample population, with disease states of similar duration/chronicity and, ideally, serial assessment of the same dogs/eyes, would further define the primary effect of the disease process, as well as the effect of secondary degenerative changes associated with chronicity, and thus aid in our understanding of the physiology of the PLR in disease states.

In summary, we demonstrated the use of chromatic pupillometry using a protocol modified for use in a heterogenous canine population without the need for chemical restraint. We demonstrated changes in pupillometry that suggested decreased ipRGC function in eyes predisposed to glaucoma, and decreased cone function in eyes with

SARDS. We suggest the use of a modified protocol for pupillometry in a veterinary clinical setting has potential to further our understanding of early glaucoma in dogs. With validation, use of pupillometry in dogs without the need for sedation or anaesthesia, may facilitate early diagnosis, and monitoring of the disease and/or response to novel therapeutic interventions in veterinary practice.

No conflicts of interest have been declared

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Figure 1. Dog's head positioned relative to the camera so that the iris plane is parallel to the camera.

Figure 2. Measurement of relative (A-B) resting and (C-D) stimulated pupil size: the relative size of the pupil (outlined in red) to the cornea (indicated by the peripheral iris margins; outlined in green) was determined by calculating the pupil diameter (yellow line) as a proportion of the corneal diameter (orange line); (C-Dc-d) to determine the degree of pupil constriction in response to a light, the same measurements were made, and the resulting relative pupil size measured as a proportion of the resting pupil size.

Figure 3. Unsuitable images for pupillometry due to: A) eyelid/dazzle reflex obscuring $>180^\circ$ of corneal circumference, and B) head and globe off-centre so that iris plane is not parallel to light source and camera

Figure 4. Relative baseline pupil sizes in normal, predisposed and SARDS eyes when stimulated with low and high intensity red light. The pupil size after stimulation is calculated relative to the baseline pupil size for each individual. The 95% confidence interval is indicated with horizontal lines. The groups between which there was a statistically significant difference are indicated with their respective p value.

Figure 5. Relative baseline pupil sizes in normal, predisposed and SARDS eyes when stimulated with low and high intensity blue light. The pupil size after stimulation is calculated relative to the baseline pupil size for each individual. The 95% confidence

interval is indicated with horizontal lines. The groups between which there was a statistically significant difference are indicated with their respective p value.

Figure 6. Relative baseline pupil sizes in normal, predisposed and SARDS eyes when stimulated with white light. The pupil size after stimulation is calculated relative to the baseline pupil size for each individual. The 95% confidence interval is indicated with horizontal lines. The groups between which there was a statistically significant difference are indicated with their respective p value.

Table 1. Comparison of subject characteristics in dogs with normal eyes, eyes predisposed to primary angle closure glaucoma and the eyes of dogs diagnosed with sudden acquired retinal degeneration syndrome (SARDS)

Table 2. Relative pupil diameter after stimulation

Supplementary table. Subject signalment

JSAP_13203_Figure 1.png

JSAP_13203_Figure 2.png

JSAP_13203_Figure 3.png

Table 1. Comparison of subject characteristics in dogs with normal eyes, eyes predisposed to primary angle closure glaucoma and the eyes of dogs diagnosed with sudden acquired retinal degeneration syndrome (SARDS)

	Normal 11 eyes	Predisposed 8 eyes	SARDS 6 eyes
Age (years)	6 (3-10)	9 (6-11)	9 (8-11)
Purebred dog	45.5% (5/11)	37.5% (3/8)	33.3% (2/6)
Sex (female)	54.5% (6/11)	75% (6/8)	50% (3/6)
IOP (mmHg)	12 (8-16)	13.5 (12-16)	11.5 (6-17)
Vision in the eye being assessed in study	100% (18/18)	100% (8/8)	0% (0/12)

Results are reported as the median (range) unless otherwise stated.

Table 2. Relative pupil diameter after stimulation

	Normal	Predisposed	SARDS	<i>p</i> value
Red light (low intensity)	0.733 ^a 0.538 – 0.995	0.748 ^b 0.661 – 0.872	0.995 ^{a,b} 0.986 – 1.00	^a 0.004 ^b 0.013
Red light (high intensity)	0.537 ^a 0.386 – 0.704	0.673 0.501 - 0.788	0.988 ^a 0.947 - 1.002	^a <0.001
Blue light (low intensity)	0.490 ^a 0.390 – 0.700	0.607 0.555 – 0.947	0.710 ^a 0.521 – 0.820	^a 0.031
Blue light (high intensity)	0.387 ^a 0.327 – 0.566	0.523 ^{a,b} 0.466 – 0.653	0.401 ^b 0.360 – 0.447	^a 0.002 ^b 0.036
White light	0.536 ^a 0.367 – 0.829	0.552 ^b 0.458 – 0.656	0.803 ^{a,b} 0.768 – 0.880	^a 0.007 ^b 0.018

Pupil sizes are recorded as relative to baseline pupil size for each eye. Results reported as median and range. Superscripts identify pairs in which there is a statistically significant difference in pupil size between groups. For each pair between which a statistically significant result was identified, the *p* value of Dunn's multiple comparisons test is listed.

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Author/s:

Graham, KL;McCowan, C;White, A

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