SALIVARY IgA: A POSSIBLE STRESS MARKER IN DOGS

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Abstract   *Animal Welfare 1995, 4: 339-350*

Stress in humans has been reported to be associated with a decrease in the salivary immunoglobulin A (s-IgA) levels enabling the possible use of s-IgA to assess stress. Prolonged stress, if reliably assessed in a non-invasive manner, may be used to assess animal welfare. This study analysed groups of dogs undergoing physical and temperamental training and s-IgA levels were measured by rocket immunoelectrophoresis in prospective samples. Behavioural assessment was carried out and cortisol levels in saliva were measured by ELISA. A significant negative correlation (P < 0.007) between the logarithmic cortisol concentrations and s-IgA levels in saliva was recorded. The behavioural assessment of the dogs agreed well with the biochemical markers. It is concluded that IgA levels in saliva may be a useful marker of dog well-being and that stress results in decreased s-IgA levels.

Keywords: *animal welfare, behaviour, cortisol, dog, salivary IgA (s-IgA), stress, well-being*

Introduction

A large number of molecules in the circulation including catecholamines, corticosteroids, endogenous opiates, and some pituitary hormones such as growth hormone and prolactin are involved in mediating effects of stressors on the immune response. These mediators, if they are to be utilized as indicators of long-term stress, generally require the animals to be restrained for blood sampling which may bias results. Other parameters of physiological changes such as heart rate or blood pressure are transient in effect and can only be complementary. Behavioural and temperamental observations can be used in an assessment of stress but they are unlikely to be standard alternatives as they require collection and processing of a large amount of data. More simple behavioural studies are biased by their subjectiveness associated with the choice of meaningful behavioural characteristics and the scoring of these. Hence it is of paramount importance to identify non-invasive approaches, where the stress markers are sensitive and the samples easy to obtain without causing unwanted and disturbing distress to the animals.

Several human studies have been carried out to examine the use of salivary immunoglobulin A (s-IgA) as an indicator of stress. Mental relaxation procedures can lead to increased secretions of s-IgA (Green *et al* 1988; Jasnoski & Kugler 1987) whereas academic stress in students may cause reduced secretion of s-IgA during their exam period

(Jemmot & Magloire 1988). A cross-sectional study in nurses indicates that anxiety can lower the levels of s-IgA (Graham *et al* 1988). Another study demonstrates that people with low scores for 'sense of humour' have a stronger negative relationship between 'hassles' and s-IgA, than those with high humour scores (Martin & Dobbin 1988). Despite the fact that predominantly chronic stress results in reduced levels of mucosal IgA, studies of pregnant women during parturition (Annie & Groer 1991) and of swimmers in training (Tharp & Barnes 1990) show that short-term stress can lead to depressed IgA levels too. Mucosal and serum levels of IgA and the control of the secretory mechanisms are recognized as independent, whereas serum and salivary levels of cortisol are correlated in man (Martin & Dobbin 1988) and in dogs (Vincent & Michell 1992).

IgA is the predominant class of immunoglobulin in a number of secretory fluids bathing mucosal surfaces, and IgA molecules found in various mucosal secretions such as saliva, milk and gastro-intestinal fluids are very similar or identical with respect to physico-chemical properties (Tomasi 1992).

The aim of the present study was to examine the potential usefulness of salivary IgA as a marker of stress in dogs, and it is the first time it has been used for animals. A decline in s-IgA levels associated with increasing stress and increased levels of cortisol was expected based on the findings in the human. We chose to analyse two groups of dogs which during their normal training and change in environment were subjected to a significant degree of stress.

Materials and methods

Dogs

Group 1: German Shepherd police dogs, Dog Training Centre, Keston, Kent, UK.
Six German Shepherd dogs (four males and two bitches) aged between twelve and eighteen months were studied. Two of them were bred on site and four were donated, including one rescued by the Royal Society for the Prevention of Cruelty to Animals (RSPCA). During the selection procedure these dogs passed the examinations for fitness, physical (including hip radiography) and temperamental suitability. All the dogs were housed in well-maintained kennels and fed once daily in the evening.

The handlers were policemen from various stations to each of whom a dog was assigned, usually well before the training. Most policemen have their dogs handed to them as 8 to 9 week-old puppies to be reared until the start of the ten-week-long training programme. Dogs usually enter the training programme when they are between 12 and 18-months-old. During the training the policemen are responsible for instructing and training their dogs through the modules of the programme.

The methods of training are based on the basic principles that will apply to any dog. These include tracking, searching and retrieving (locating hidden objects and persons), agility testing and recovering hidden objects marked with human scent. The trainer performs a close study of his animal's attitude, behaviour, and the speed of learning and then designs his programme accordingly. Spontaneous and strenuous but gentle commands with frequent rewards and copious amounts of praise are used to obtain a successful outcome.

In this study the following six dogs were allocated for the initial training, four of them completed the schedule successfully, and the other two had to be withdrawn due to

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unsuitability (see results for details). The stressfulness of the training programme varies between dogs. Confident and robust dogs well adapted to the environment often seem enthusiastic throughout the programme. In these cases the training programme may be considered as a positive stress for the dogs as described by Wiepkema & Koolhaas (1993). However, timid dogs and dogs where the dog–handler relationship is not properly developed may find the training programme stressful and the stress will increase during the programme. Consequently police dogs under training were considered to be ideal for the present study because they are closely monitored throughout the training programme. It was expected that confident dogs with a well-established dog–handler relationship would enjoy the training. These dogs were thus expected to have relatively low salivary cortisol levels. Dogs which were more nervous and timid, and without a firmly established dog–handler relationship were expected to find the training more stressful. These dogs were thus expected to have relatively high salivary cortisol levels.

Dog 1 was a donated male dog, 11-months-old when the training started. He had his dew claws of the hind legs removed and passed the screening satisfactorily.

Dog 2 was a bitch, bred on site, and aged 13-months. Her father was a good dog but had a submissive character and the mother was a very aggressive working dog.

Dog 3 was a male dog bred locally and 12-months of age. His father was very active whereas his mother was of average quality.

Dog 4 was a male dog with a sad past life. He was rescued by the RSPCA from cruel treatment and was about 17-months of age. He qualified in all the tests easily and his agility was extraordinary. It was anticipated he would be very useful as a police dog.

Dog 5 was a 13-month-old, donated, male dog and he was expected to perform well in the training programme.

Dog 6 was a bitch aged 10-months and had spent only one week in the centre since being donated and she had not met her handler until a day before the start of the training. She performed well on all the tests and had an excellent hip anatomy.

Group 2: army dogs, Defence Animal Centre, Melton Mowbray, Leicestershire, UK.
This group consisted of 10 army dogs, donated by owners. They included German Shepherds, English Springer Spaniels, and a Labrador. All dogs in this group were exposed to a novel environment considered stressful to the animals. The dogs were brought to the Central Army Unit where a general health examination and hip radiography were carried out to identify potentially useful dogs. The dogs were normally then sent within three weeks of receipt, to institutions such as Dogs for the Blind, and Dogs for the Police etc.

Each batch of dogs was housed in a common pound with some dogs in individual kennels within the pound and others tied to interior boundary walls in a totally open environment. The dogs were fed twice a day. All the dogs were in an entirely new social environment as reflected by the disturbance of all members of the pound in response to simple stimuli. Two saliva samples were collected, one during examination just after their arrival, and the other ten days later.

Saliva sampling

Samples were collected as each dog was brought to the adjoining veterinary examination room between 0830 and 0900h. All dogs were familiarized with the procedure during previous visits before the initial collection day. Routinely, any deviations from normal health or behaviour were noted and the mouth was examined for oral health and food contamination. Two loose balls of absorbent cotton wool (Boots Co Ltd, UK) approximately 2cm in diameter were left under the dogs tongue and the jaws were held closed for 30 to 45 seconds. Contaminated balls were discarded. Before removal the balls were used to wipe the inner surface of the lips. The balls were immediately placed in the barrel of a 2ml syringe and the saliva squeezed out into an Eppendorf tube maintained at 4°C. Tubes were taken to the laboratory the same day, spun at 2800rpm for 20 minutes, and the supernatant separated and volume marked. One hundred microlitres of each sample were transferred to smaller tubes and were frozen at -20°C for cortisol assay. Twenty microlitres were used for immunoelectrophoresis, and to the remainder was added 5 microlitres 0.1% sodium azide before storing the tubes at 4°C.

Reagents

All chemicals used were purchased from Sigma Chemical Co Ltd (Poole, UK) or BDH (Merck Ltd, Lutterworth, UK) and were of analytical reagent grade wherever possible. The antibody was obtained from Nordic Immunological Laboratories (Tilburg, The Netherlands).

IgA rocket immunoelectrophoresis

IgA was quantified by rocket immunoelectrophoresis (Laurell 1972), a standard technique, using rabbit anti-dog IgA at a concentration of 1:75 (antibody:gel). Inter-assay and intra-assay variations were below 10 per cent.

Serum collected from four healthy dogs was pooled and used as the standard, and arbitrarily assigned to contain 100 Arbitrary Units(AU) of IgA. Standard dilutions were obtained by carrying out two-fold serial dilutions and the final concentrations of each tube remained as 100, 50, 25, 12.5, 6.25, 3.125 AU ml^{-1}. These standards were run with saliva samples in each assay and their rocket heights used to produce a standard curve which was used to measure the IgA levels in samples. The regression coefficient (r) for the curve for each plate varied between 0.9992 and 0.9999. Dilutions measured against sample rocket heights on the curve were antilogged to determine the IgA concentration in arbitrary units.

Cortisol ELISA

Cortisol levels in all samples were measured using an ELISA kit (Cortisol Serozyme, Serono, from Intersep, Wokingham, UK). All samples were run in duplicate. The interassay variation levels were between 0.1 per cent and 10.5 per cent with a mean of 6.2 per cent.

Behavioural scoring scheme

A scoring scheme was developed for behavioural assessment of the police dogs by their individual handlers and their instructor (Figure 1). Each character was rated and scored accordingly (3, 0, or -3) depending on the dog's behaviour with respect to the individual characters: 3 being the most positive scoring for all characters and -3 being associated with a dog exhibiting stress. Dogs 5 and 6 were not behaviourally assessed because they had spent too little time with their handlers prior to training to allow a reliable assessment.

ASSESSMENT OF DOG CHARACTER

DOG'S PERSONALITY
- ☐ CALM
- ☐ MODERATELY CALM
- ☐ NERVOUS

DOG'S RESPONSE TO TRAINING
- ☐ WELL DISCIPLINED
- ☐ FAIRLY WELL DISCIPLINED
- ☐ POORLY DISCIPLINED

DOG'S RESPONSE TO HUMANS
- ☐ CONFIDENT
- ☐ CAUTIOUS
- ☐ FEARFUL

DOG'S RESPONSE TO SAME SEX DOG
- ☐ CONFIDENT
- ☐ CAUTIOUS
- ☐ FEARFUL

DOG'S ACTIVITY LEVEL
- ☐ CALM
- ☐ ACTIVE
- ☐ HYPERACTIVE

DOG'S RESPONSE TO NOVEL STIMULI
- ☐ CONFIDENT APPROACH
- ☐ CAUTIOUS APPROACH
- ☐ FEARFUL APPROACH

DOG'S FITNESS
- ☐ GOOD
- ☐ FAIR
- ☐ POOR

DOG'S RESPONSE TO TRAINING
- ☐ GOOD (UNSTRESSED)
- ☐ FAIR (STRESSED BUT COPED)
- ☐ POOR (VERY STRESSED)

Figure 1   Behavioural assessment form used for police dogs (group 1).

Statistics
Group 1, police dogs
A general negative semi-logarithmic correlation between levels of cortisol and IgA in saliva was observed in the individual dogs, allowing all data to be pooled for the graph (Figure 2) and a linear regression analysis carried out. An ANOVA test was performed to analyse intra-individual and inter-individual variation in salivary IgA and cortisol levels.

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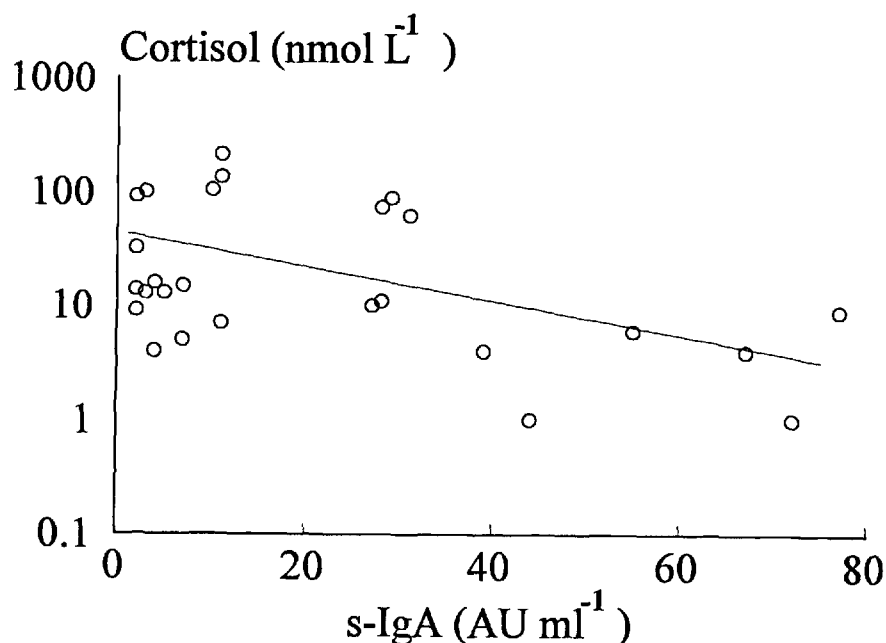
Figure 2   Levels of cortisol and IgA in saliva samples from police dogs (group 1).

Group 2, army dogs
A non-parametric Wilcoxon test was employed to analyse the significance of the expected decline in s-IgA levels with time.

Results and Discussion

Group 1, police dogs
Dog 1 performed very well despite a lower than normal body-weight for his age, perhaps due to previous under-nourishment. The transfer to the police handler resulted in major improvements to his size and nature. His capacity in all aspects of learning was excellent. His adaptive ability was associated with consistently high IgA levels in saliva during the training, while other dogs exhibited fluctuations (Table 1). It was unfortunate that Dog 1 was not available when the fifth samples were obtained from the other dogs to confirm his overall capability, as shown by his fourth and sixth samples, in which none of the other dogs could match him. The cortisol levels remained far below average, except for the initial sample, confirming his very low stress level throughout the training (Tables 1 and 2).

Dogs 2 and 3 were born and bred in the centre up to eight-weeks-old and then cared for by the handlers. They were expected to experience very few, if any, problems with relationship or adaptation in the course of the training. They both showed a similar s-IgA profile, though Dog 3 had an initially high level (Table 1). Both of them performed very well despite a poor start by Dog 2. However, she had a consistently good learning ability throughout the initial few weeks of the exercise. On the third sample only Dog 2 matched Dog 1 with Dog 3 slightly lower and all others far below with regard to s-IgA level and training performance (Table 1).

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Dog 4 was recovered by the RSPCA from a home where he was cruelly treated and reacted below average with regard to performance during training. He had poor perceptive capacity, and showed a hyperactive responsiveness to novel stimuli, which may be a quality of his learned greater alertness, but whether he could be a successful police dog remained to be seen. He was poorly adapted to the increasing load of training and the third sample had a very low s-IgA level (Table 1). Dog 4, like Dog 5, had been acclimatizing very well as judged by his behaviour by the end of the first 3 days training even though the cortisol levels were increasing at that time. An explanation is not readily available for this inconsistency. Dog 4 continuously required firm and repeated commands to be focused on the training.

Dog 5 was in good physical condition and lively, but the first saliva sample showed low IgA levels. His initial learning capacity was satisfactory but as the intensity of training slowly built up he was, at times, inclined to manifest a behaviour of circling while trying to bite his tail. In these circumstances he was totally disobedient. Dog 5 was expected to recover with time, but the behaviour persisted with increasing frequency as the intensity of the training peaked. After careful consideration of his possible future failure to satisfy demands, he was withdrawn from the group. However, he was a good pet and was soon rehabilitated in a caring home. Although Dog 5 showed a gradual increase in s-IgA during the first 10 days, the fourth sample demonstrated an acute drop compared to all others (Table 1). This agrees well with the degree of stress he had been experiencing. The fifth sampling day was the second day after his withdrawal and showed levels as low as Dog 4. On the last sample, Dog 5 was the only dog with a reasonably high s-IgA level, apart from Dog 1, possibly due to a week long rest, whereas all the other dogs were under training and stress.

Table 1 **Salivary IgA (AU ml^{-1}) and Cortisol (nmol L^{-1}) concentrations in police dogs (German Shepherd Dogs).**

Dog number	sex	age months	parameter	day 1	day 4	day 8	day 11	day 18	day 25
1	male	11	IgA	31	2	77	67		182*
			Cortisol	62	32	9	4		15
2	female	13	IgA	4	11	72	11	4	2
			Cortisol		215	1	7	4	9
3	male	12	IgA	3	2	55	44	2	7
			Cortisol	100	91	6	1	14	5
4	male	17	IgA	7	28	14	27	5	4
			Cortisol	15	73	794**	10	13	16
5	male	13	IgA	4	29	39	4	3	28
			Cortisol		88	4	16	13	11
6	female	13	IgA	10	11				
			Cortisol	104	136				

* Value calculated from extrapolation of standard curve and not used in statistical analysis.
** Value based on high dilution of sample and not used in statistical analysis.

Dog 6 was immature and had spent only a week with the handler after being donated to the police. She passed the screening quite well although the dog–handler relationship was not well established before the start of training, and hence she responded poorly to the commands. By the end of two weeks she was withdrawn from the course to provide sufficient time with her handler before joining the next batch of dogs for training. The only two samples available from her had high cortisol and low s-IgA levels which seemed to confirm her unsuitability (Table 1).

The results of the behavioural assessment and the mean s-IgA concentrations for the police dogs are shown in Table 2. Low levels of s-IgA were recorded in dogs with a low behavioural score, and high levels of s-IgA were associated with a high behavioural score. This result is in good agreement with the hypothesis of using s-IgA as a marker of well-being in the dog. The assessment of behaviour was done towards the end of the training, but the results apply to the general character of the dogs. Dogs 5 and 6 were not scored because there was not a sufficiently intimate knowledge of their background and previous behaviour.

These results indicate that a few consecutive measurements of s-IgA at the beginning of the training schedule may aid in predicting how the dog is going to cope with the subsequent training.

Table 2 A comparison of IgA (AU ml^{-1}) concentrations and behavioural scores in police dogs. The behavioural score is the sum of the scores with respect to eight behavioural characters (Figure 1). The maximum score is 24 and the minimum score is -24. Dogs 5 and 6 were not available for a full objective assessment.

Dog	IgA mean (AU ml^{-1})	Number of samples	Behavioural scores
1	44	4	15
2	17	6	12
3	19	6	12
4	14	6	9

In the individual police dogs a negative semi-logarithmic correlation between salivary cortisol and IgA was generally observed. Figure 2 shows the relationship between salivary IgA and cortisol concentrations in individual samples of all police dogs. A significant negative linear semi-logarithmic correlation (r = -0.46) between cortisol and s-IgA levels (*P* < 0.007, one-tailed test) was recorded.

The mean inter-individual variation between dogs with respect to s-IgA and cortisol levels was 538 per cent and 239 per cent, respectively. The mean intra-individual variation within the individual dogs with respect to s-IgA and cortisol was 1997 per cent and 5514 per cent, respectively. These results show that both s-IgA and cortisol are dynamic parameters, and that the inter-individual variation with time during training is greater for IgA than for cortisol. This may indicate that s-IgA may be very useful to discriminate between stressed

and unstressed dogs. By contrast, the intra-individual variation for IgA is lower than for cortisol. Corticosteroids have frequently been used as stress markers, but they exhibit great intra-individual variation and fluctuate with diurnal and other biological rhythms. In dogs a diurnal variation of glucocorticoid levels may not be as apparent as in other species (Johnston & Mather 1978). Glucocorticoid levels are generally considered poor indicators of chronic stress since they usually decrease to baseline within hours after an initial rise (Manser 1992). However, in a recent study of cats stressed for 21 days by altered caretaking Carlstead *et al* (1993) found urinary cortisol concentrations to be consistently elevated throughout the three-week period. Salivary cortisol levels in the dog have been shown to increase from 5.3 nmol L^{-1} to 41.9 nmol L^{-1} following injection of ACTH (Vincent & Michell 1992). The cortisol levels recorded in the present study seemed generally to be within the normal range, and the recording of few very high levels indicate that a maximal or 'ceiling' level was not reached in the present study. Cortisol inhibits immunoglobulin synthesis and this suggests the potential usefulness of secretory immunoglobulins, which are large protein molecules with a long half-life, as more stable stress markers.

In the human s-IgA levels have been reported to be influenced by the collection method (Aufritch *et al* 1992). In the present study sampling was carried out at the same time on every occasion using a standardized sampling procedure. German Shepherd dogs have been reported to have relatively low serum IgA levels (Whitbread *et al* 1984), but serum levels of IgA are not correlated with saliva levels (Tomasi 1992). Since comparisons between breeds were not the purpose of the present study, potential differences between breeds were not addressed. Bacterial protease activity has been shown to be responsible for denaturation of the salivary proteins including IgA in the oral cavity. The importance of collecting saliva direct from the parotid duct has been emphasized in experimental dogs to avoid enzymatic hydrolysis of IgA (Phillips *et al* 1983). This, however, requires anaesthetizing the patient and contradicts the aim of the present study, which focuses on non-invasiveness. Studies carried out in our laboratory demonstrated no significant decrease of s-IgA in samples stored at room temperature and at 4°C for up to 48 hours. However, all samples were stored at -20°C until analysis, as a routine practice.

Group 2, army dogs

Ten army dogs were sampled on arrival at the army kennel and 10 days later. Table 3 shows the decrease in s-IgA recorded in all army dogs. The mean s-IgA value of the 10 dogs present on Day 10 decreased from 143 ± 83 SEM to 4 ± 0.7 SEM (one-tailed *P* value 0.001) during this period.

In the army dogs very large inter-individual variation in salivary s-IgA levels was recorded immediately after they were brought from private homes into the army kennel. The environmental change they experienced from the time they left their homes had presumably not had sufficient time to produce an inhibition of s-IgA synthesis. The second samples obtained 10 days later were significantly lower and may reflect the severe stress they were facing in a large noisy common pound. The extreme change and the dramatic drop of s-IgA exhibited by all dogs analysed indicates a good correlation between stress and inhibition of s-IgA.

Table 3   **Salivary IgA concentrations in army dogs at intake and 10 days later.**

Dog	Breed	Sex	Age months	Salivary IgA (AU ml⁻¹) at intake	Salivary IgA (AU ml⁻¹) 10 days later
Jason	GSD	male	18	11	5
Wally	GSD	male	30	33	3
Toby	ESS	male	30	5	3
Charlie	Lab	male	12	283	3
Honey	ESS	female	30	108	5
Duke	GSD	male	36	54	2
Pip	ESS	female	24	20	2
Trickle	ESS	female	12	47	7
Chako	GSD	male	12	855	9
Zeus	GSD	male	25	17	3

GSD – German Shepherd dog; ESS – English Springer Spaniel; Lab – Labrador Retriever.

Animal welfare implications

The present study for the first time demonstrates a significant negative correlation between concentrations of cortisol and IgA in the saliva of dogs. This suggests the potential of using IgA as a marker of stress and well-being in dogs.

In conclusion, the present study strongly suggests a negative association between long-term stress and salivary levels of IgA in the dog. Salivary cortisol levels were found useful too as an independent marker supporting behavioural analysis. To confirm the usefulness of s-IgA as a marker of well-being in dogs, studies of working dogs including frequent sampling and collection of behavioural data are essential. Once these comprehensive studies are accomplished it will perhaps be possible to use s-IgA as a measure of well-being in dogs and as a reliable stress indicator.

Acknowledgements

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