Dog appeasing pheromone®:

A useful tool to minimize stress and aggression of African wild dogs (*Lycaon pictus*)?

Minor research project:

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Abstract

Dog appeasing pheromone (DAP®) could be an effective method or additional tool to reduce stress and intra-pack aggression of captive held African wild dogs (*Lycaon pictus*) during translocations and pack formations. In this study three experiments were conducted, to evaluate the perception of DAP® by African wild dogs, to determine the effect of DAP® on stress levels of captive African wild dogs and to evaluate the effect of DAP® on intra-pack aggression and social integration during regrouping in captivity. The findings of these experiments indicate that 1) behavioural responses towards areas treated with DAP® or placebo spray, while tested in an outside area, are indicative, although insignificantly in the sample size of this study, for the perception of DAP® by African wild dogs. Importantly wild dog packs, in general showed more interest for areas treated with DAP® which is promising, 2) an cortisol-3-CMO EIA is suitable for reliably monitoring changes in adrenocortical activity via measures of glucocorticoid metabolites (GCM) concentrations in the faeces African wild dogs, 3) changes in adrenal functioning after adrenal stimulation are reflected by changes in faecal GCM concentrations which demonstrates the physiological relevance of faecal GCMs as indicator of adrenocortical activity for African wild dogs, 4) DAP® collars substantially reduce baseline stress levels of female African wild dogs. 5) relative low frequencies of severe and ritualized aggression are present during regrouping events when DAP® spray and DAP® collars are applied before regrouping and 6) the strength of (existing) resting relationships between pack members is not affected.

Keywords Adrenocorticotropic hormone (ACTH), African wild dog, Dog appeasing pheromone (DAP®), Enzyme immunoassay (EIA), Faecal glucocorticoid metabolites, Intra-pack aggression, *Lycaon pictus*, Pack formation, Translocation, Social introduction, Stress

Abbreviations

ACTH	Adrenocorticotropic hormone
ADL	Adult dog length
DAP®	Dog appeasing pheromone
EIA	Enzyme immunoassay
GCM	Glucocorticoid metabolites
RIA	Radioimmunoassay

1. Introduction

African wild dogs (*Lycaon pictus*) are highly social, communally hunting carnivorous mammals that live in small cohesive packs. A typical stable wild dog pack comprises a dominant breeding pair (alpha pair), a number of related subordinate non-breeding adults and their dependent offspring. Within a wild dog pack all males are related to each other and all females are related to each other as well. The highest-ranked male and female breed and inhibit reproduction by subordinates, although occasionally subordinate males reproduce and subordinate females become pregnant. Subordinate wild dogs have an important role in raising the pups of the alpha pair. The subordinates help by means of protection against predators and providing food (Creel and Creel, 2002; Woodroffe et al., 1997). African wild dogs rely on cooperative hunting to obtain food and assistance of subordinates is essential for raising offspring (Derix, 1994; Gusset et al., 2006). The need for a social stable cohesive pack, characterised by strong social relationships is therefore high.

The African wild dog, which is only found in South East Africa, is classified as an endangered species by the Species Survival Commission (SSC) of the International Union for Conservation of Nature (IUCN) (McNutt et al., 2011). A continuing decline in the numbers of mature individuals is observed and there are no subpopulations estimated to contain more than 250 mature individuals.

In 1997 a status, survey and conservation action plan was compiled by the IUCN/SSC Canid Specialist Group for the in situ conservation & recovery of the African Wild Dog. One of the conclusions of the IUCN/SSC Canid Specialist Group was that meta-population management would be a useful strategy to maintain small sub-populations in highly fragmented landscapes e.g. in Southern Africa and that reintroduction could be used to establish a network of small subpopulations where funds are available (Woodroffe et al., 1997). In South Africa, a metapopulation management programme for the African wild dog was launched in 1998, which involves the re-introduction of wild dogs into suitable conservation areas and periodic translocations among a network of small subpopulations to maintain genetic diversity in geographically isolated conservation areas (Mills et al., 1998 as cited in Gusset et al., 2006). In addition, ex situ conservation breeding programs for the African wild dog were established in 1990 by EAZA, the European Endangered species Programme (EEP), and in 1991 by AZA, the Species Survival Plan (SSP).

The goal of the in situ meta-population management programme and ex situ conservation breeding programs is to ensure the sustainability of a healthy genetically diverse and demographycially varied population of African wild dogs. To maintain the viability (genetic diversity) of small in situ and ex situ subpopulations of African wild dogs, frequent translocation, group enlargement, group merging, and formation of new groups is needed. For successful translocations and reintroductions of artificial created packs, comprising wild caught and/ or captive-bred / raised African wild dogs formation of social stable cohesive pack in captivity is essential. Social bonding of captive-bred / raised with wild-caught animals increases the survival chances of captive-bred / raised animals in the wild after release (Gusset et al., 2006; Woodroffe et al., 1997).

Free-living African wild dogs form new packs when small sub-groups of the same sex, usually juvenile siblings, leave their natal group join other migrating sub-groups of the opposite sex (Woodroffe et al., 1997). When these unrelated migrating sub-groups of the opposite sex join, they undergo a period of ‘trial courtship’ first, which may result in a stable reproductive unit (Frame et al., 1979; McCreery, 2000). Direct after the formation of a new pack the wild dogs establish a dominance hierarchy. Dominance interactions that occur during this period of ‘trial courtship’ may already be decisive for the access to mates and can therefore be rather aggressive and accompanied by severe fighting (Derix, 1994; Frame and Frame, 1976; Frame et al., 1979; McCreery, 2000; van Lawick-Goodall and van Lawick-Goodall, 1970). After these decisive conflicts the males comply with their status as breeders or non-breeders as long as the social circumstances remain unchanged (Frame and Frame, 1976). In social stable cohesive packs most conflicts are settled by ritualized displays or ritualized (mild) aggression (Creel et al., 1997). The stability and cohesion within established pack appears to be maintained largely through amiable and submissive interactions.

In captivity however, one of the main problems associated with translocations, group enlargement, group merging and group formation of African wild dogs is intra-pack aggression (Hofmeyr, 1997 as cited in Gusset et al. 2006; Gusset et al., 2006; Gusset et al., 2008; Kock et al., 1999 as cited in Gusset et al. 2006; McCreery and Robbins, 2004; McCreery and Robbins, 2005; McCreery and Robbins, 2006; McCreery and Robbins, 2007; Scheepers & Venzke, 1995 as cited in Gusset et al. 2006; pers. comm. H. Verberkmoes, (EEP coordinator)). This level of intra-pack aggression is rarely observed in the wild and can result in serious injury with mortality as consequence in a non-well documented number of cases (pers. obsv. Author; McCreery and Robbins, 2004; McCreery and Robbins, 2005; McCreery and Robbins, 2006; McCreery and Robbins, 2007; pers. comm. A. van Dyk; pers. comm. H. Verberkmoes, (EEP coordinator)). Gusset et al. (2006) suggests that intra-pack aggression under captive conditions can be viewed as a struggle for dominance, which ensures access to mates. Stress is however another important factor which may contribute to the occurrence of intra-pack aggression.

During translocations, group enlargement, group merging and group formation there are many potential sources of stress (Teixeira et al., 2007; Waples and Gales, 2002) and the accumulative effect of these physical, physiological and psychological stressors can result in intra-pack aggression during pack formation and lead to social instability of the pack after formation.

Most research regarding the behavioural and physical stress response of animals after group enlargement (Alberts et al., 1992; Bernstein, 1964; Bernstein et al., 1974[a]; Goo and Sassenrath, 1980; Gust et al., 1992; Kaplan et al., 1980; Schaffner and French, 1997; Southwick, 1967) group merging (Bernstein et al., 1974[a]; Bernstein et al., 1974[b]; Bernstein et al., 1979; Clarke et al., 1996; Rose et al., 1972; Seres et al., 2001; Southwick, 1967) and group formation (Bernstein and Mason, 1963; Bernstein et al., 1979; Capitanio et al., 1998; Clarke et al., 1995; Gust et al., 1996; Mendoza et al., 1979) has been conducted on non-human primate species (See for a detailed review, Honess and Marin, 2006)). In the majority of these studies an increase in aggressive behaviour and / or basal cortisol levels were observed in response to the change in social conditions. Under social instability higher aggression levels and / or basal cortisol levels were found (Cavigelli and Pereira, 2000; Coe et al., 1979; Keverne et al., 1978; Mendoza et al., 1979; Rose et al., 1975; Sapolsky, 1983; Sapolsky, 1987; Sapolsky, 1991; Sapolsky, 1992; Sapolsky, 1993).

Despite its being generally accepted that artificial pack formation in African wild dogs in captivity is cumbersome, very few studies investigated how artificial pack formation, translocations and reintroductions of (artificial created) African wild dog packs can be improved. Evaluations of release events of African wild dogs indicate that wild dog packs kept in a pre-release holding facility (boma) for a period of time before release enhance re-introduction success (Gusset et al., 2006; Gusset et al., 2008). In the majority of the evaluated release events wild dogs were kept in the same boma from the beginning (Gusset et al., 2008). This is in accordance with Hofmeyr (2001) (as cited in Gusset et al., 2008; Lines, n.d.) who suggests that all animals from the same sex must be introduced at the same time to prevent fighting and that opposite-sex groups should be introduced into the same boma from the start, as the groups should not know each other. However, in 50% of the evaluated release events aggression was observed in the boma (Gusset et al., 2008).

Results of Gusset et al. (2006) and Graf et al. (2006), on the other hand, suggest that a period of social acclimatization, by keeping wild dogs in adjacent bomas, before they are united, may increase the likelihood of successful social integration. They hypothesize that such a social acclimatization period before pack formation allows wild dogs to establish first contacts and may already induce the establishment of social bonds. This social acclimatization period before pack formation may mimic the natural situation of 'trial courtship' in which new packs form over a period of several days (Frame et al., 1979; McCreery, 2000). Although the results of Gusset et al. (2006) and Graf et al. (2006) are not conclusive yet, they indicate that the social acclimatization period before pack formation promotes social integration and the formation of a social stable cohesive pack.

In mammals, prior familiarity between individuals before introduction seems to be an important universal factor that increases the likelihood of successful social introduction (Powell, 2010). Primate studies show that previous social familiarity between individuals involved in group enlargement, group merging and group formation reduces diminish stress and aggression levels during these events (Bernstein et al., 1979; Rosenblum and Lowe, 1971; Williams and Abee, 1988). A common applied strategy in primate group enlargements is to introduce new animals to individuals or subgroups of the established group first, prior to introduction to the entire group (Alford et al., 1995; McDonald, 1994; Meshik, 1999).

However, this strategy is not applicable for all social mammalian species. Temporal absence of members of an established group may disrupt existing hierarchical bonds between members and can cause instability of the entire group which may result in significant stress, fighting and injury. Another common applied approach to familiarize new individuals with each other before social introduction is establishment of non-tactile sensory contact and limited tactile contact across a barrier (e.g a meshed fence) prior to full contact physical introduction. Such phased / stepwise introductions have been successfully used in a variety of mammalian species (e.g. African elephant (*Loxodonata Africana*) (Burks et al., 2004); clouded leopard (*Neofelis nebulosa*) (Law and Tatner, 1998) and maned wolf (*Chrysocyon brachyurus*) (Bestelmeyer, 1999) (See for a detailed review, Powell, 2010).

For the introduction of African wild dogs, establishment of non-tactile sensory contact could be a useful strategy to familiarize individuals or subgroups. A social acclimatization period in adjacent bomas before pack formation, in which visual, audial and olfactory contact can be established, may considerably reduce potential stress and aggression during group enlargement, group merging and group formation and prevent injury and mortality.

European zoos have tested the application of sedative pharmaceutical drugs, *e.g.* Fluoxetine hydrochloride (trade names Prozac, Fontex, Ladose, Sarafem, during several introductions and translocations of African wild dogs, to prevent conflict situations and to suppress aggression and physiological and psychological stress reactions during these events (pers. comm. H. Verberkmoes, (EEP coordinator)) However, sedation did not produce the desired effect in the majority of cases. This seems similar to findings in other species. For example, application of chlordiazepoxide (Librium) in chimpanzees, to lower initial excitement and avoid aggression during group formation, did not produce the desired effect (van Hooff, 1973). Sedative drugs only suppress and do not overcome, treat or prevent the underlying arousal, social stress and/or fear and anxiety and may in addition have a disorienting effect. Application of sedative drugs is therefore not without risk and has only a short term effect.

In conclusion, severe intra-pack aggression in African wild dogs observed during translocations and pack formation under captive conditions hinders maintenance of viable (genetic diverse) of small in situ and ex situ subpopulations. The methods that have been applied so far to prevent or reduce stress and intra-pack aggression and/or facilitate social integration, e.g. the use of sedative drugs and social acclimatisation, do not overcome all problems or have negative side effects.

The application of Dog appeasing pheromone ® (DAP®; Ceva Sante Animale, Libourne, France) instead of sedative drugs could be an effective and more gentle alternative to diminish stress and intra-pack aggression during translocations and pack formation as seen in canid species such as the African wild dog and may facilitate social integration and solve reported behavioural problems.

Pheromones are mixtures of volatile components perceived by mammals through either the main olfactory epithelium or the vomeronasal organ. DAP® is an artificial congener of the natural form of dog appeasing pheromone excreted by the domestic dog (*Canis lupus familiaris)*. It is a synthetic mixture of fatty acids based on those that have been identified from the secretions of sebaceous glands in the intermammary sulcus of bitches shortly after parturition (Mills et al., 2006; Pageat and Gaultier, 2003). The biological function of the natural form is to calm and reassure (appease) new born puppies 2-5 days after parturition until weaning (Denenberg and Landsberg, 2008; Pageat and Gaultier, 2003). DAP® is not a cerebral suppressant; it does not have the side effects of sedative pharmacological agents and has been proposed as a 'natural' way to appease domestic dogs (Tod et al., 2005). Several studies showed that adult domestic dogs are still susceptible for these maternal pheromones. Dog-appeasing pheromone has been reported to reduce fear in puppies in a new environment (Taylor and Mills, 2005), anxiety and stress during transportation (Gandia Estellés and Mills, 2006; Gaultier and Pageat, 2003) and signs of fear or anxiety in veterinary settings (Mills et al., 2006). Gaultier et al., 2005 found, in addition, no significant difference in effectiveness of DAP® and clomipramine for separation-related behaviour problems in domestic dogs. DAP® may also diminish anxiety of aggressive dogs in the veterinary practice (Mills and Hargrave, 2004). However, Mills et al. (2006) found no evidence that DAP® has an effect on aggression in veterinary settings.

In mammals, appeasing pheromones consist of three fatty acids, which are always associated in the same ratio and could be considered as the "mammal appeasing message", and some other components, which could be considered as the species specific message (Pageat and Gaultier, 2003). Gaultier et al. (2005) hypothesize that a high concentrated solution of pheromone specific for one species could have an expected effect on a closely related species. Some preliminary (uncontrolled) cases studies indicate that wild species are susceptible for pheromones of closely related domestic species (Gaultier et al., 2005; Pageat). A preliminary (uncontrolled) case study on the maned wolf for example suggests an appeasing effect of DAP® during transport. Some other preliminary (uncontrolled) case studies on the panther (*Panthera pardus*), tiger (*Panthera tigris*) and Asian elephant (*Elephas maximus*) suggest an appeasing effect of artificial appeasing pheromones during transport and introduction events (Gaultier et al., 2005; Pers. comm. P.Pageat).

DAP® could therefore be an effective alternative or an additional tool to reduce aggressive interactions and social stress within wild dog packs and/or could facilitate social integration. DAP® could decrease the level of inter-pack aggression and social stress and improve socialization and habituation stress and consequently reduce the risk of physical injury and mortality during and after pack formation.

The aim of the current project is to determine the effect of DAP® on stress levels of captive African wild dogs and to evaluate the effect of DAP® on intra-pack aggression and social integration during regrouping in captivity. This will be done by measuring behavioural and physiological parameters. In summary, this study had four objectives:

1) The first objective (experiment 1) is to evaluate if African wild dogs perceive DAP® spray by comparing the behavioural response towards areas treated with DAP® or placebo spray. The hypothesis is that African wild dogs show more interest for areas treated with DAP® and will rest longer close to these areas

2) The second objective (experiment 2a) was to test whether changes in adrenocortical activity in African wild dogs, reflected by glucocorticoid metabolite (GCM) levels in the faeces, can be reliably monitored by using an cortisol-3-CMO enzyme immunoassay (EIA). An adrenal stimulation test (ACTH Challenge test) was performed to validate the EIA for monitoring faecal GCM levels and to demonstrate physiological relevance of GCM levels as indicator of adrenocortical activity for African wild dogs.

3) The third objective (experiment 2b) was to determine the effect of DAP® collars on baseline stress levels of single housed captive African wild dogs. The hypothesis is that application of DAP® collars reduces baseline stress levels of the single housed African wild dogs after application. A non invasive faecal sampling technique was used to determine stress levels. Faecal GMC concentrations, which were used as indicator of stress, were quantified with an cortisol-3-CMO EIA.

4) The fourth objective (experiment 3) was to examine the effect of the application of DAP® on the behavioural process of regrouping. The hypothesis is that application of DAP® spray and DAP® collars before regrouping leads to a smoother establishment of the new formed pack, characterized by low levels of sever aggression and faster and/or better social integration

The results of this study could have important implications for Zoos and breeding centres. When results indicate that the application of DAP® is effective, DAP® should be considered as a useful tool to reduce social stress and/or aggressive interactions during and after pack formation in captivity. Successful pack formation is important for the reintroduction of captive bred wild dogs from breeding centres and to re-establish populations in endangered areas. In addition DAP® could be applied during animal transport, and can enhance the efficiency of costly, translocations during reintroduction operations of captive and free-living African wild dog packs.

2. Materials and methods

2.1 Subjects, study site, and general husbandry

The 19 captive wild dogs involved in this study (Table 1) were housed individually or in small groups of 2 to 3 individuals at the Ann van Dyk Cheetah Centre - De Wildt which is located near Brits in North-West Province, South Africa. Information on sex, age, and genealogical relationship within the packs is provided in Table 1.

The wild dogs were kept in outdoor enclosures which were fenced with wire mesh and covered with natural vegetation, e.g. grass, bushes and trees (Fig. 1). The enclosures had 2 corner camps which were separated by a crush cage (Fig. 2). The corner camps and crush cage were used for sample collection, drug administration, immobilisation and to capture the study animals in transport boxes. All study animals had audio, visual and olfactory contact with dogs in neighbouring enclosures and were exposed to ambient environment conditions.

The African wild dogs were group fed, daily, 6 days a week and subsequently starved for 1 day. Their diet consisted of pieces of raw horse meat on the bone and of pellet shaped domestic dog food from Eukanuba® (The Iams Company, Dayton, Ohio, USA). Water was available *ad libitum*. Two times a week, water mangers were cleaned, water was refreshed and faeces and remaining bone material was removed from the enclosures by the animal keepers while the dogs were in the main or corner camp. The presence of staff in the enclosure was part of the general husbandry procedure so the study animals were already habituated to this situation at the onset of the study

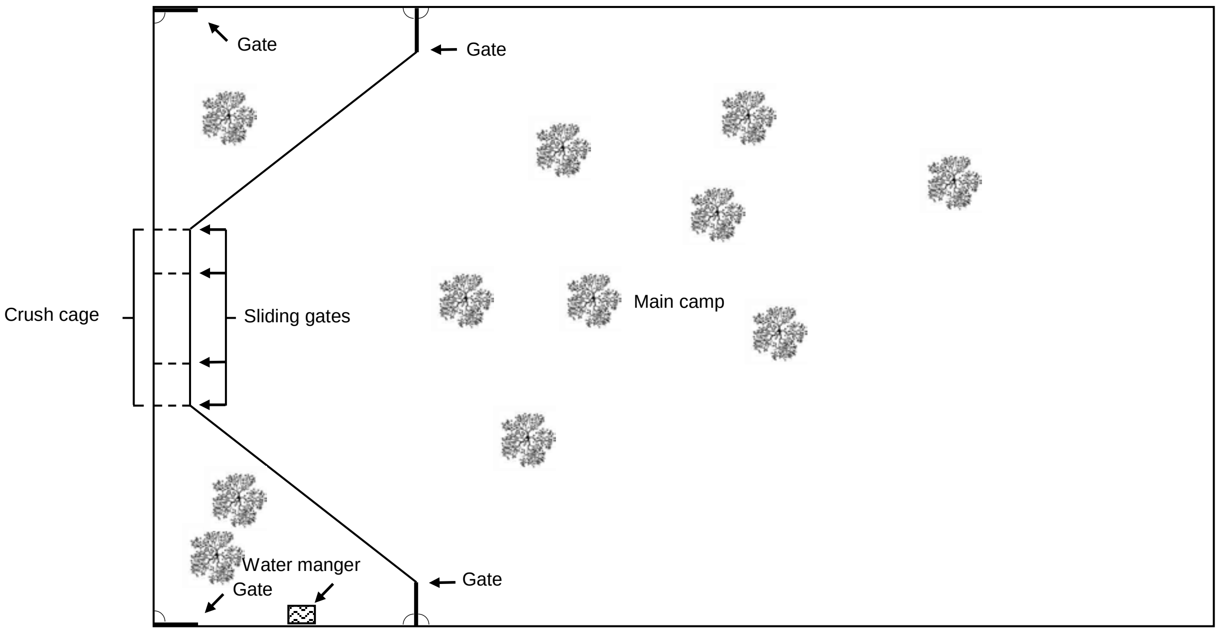

Figure 1. General layout of the enclosures

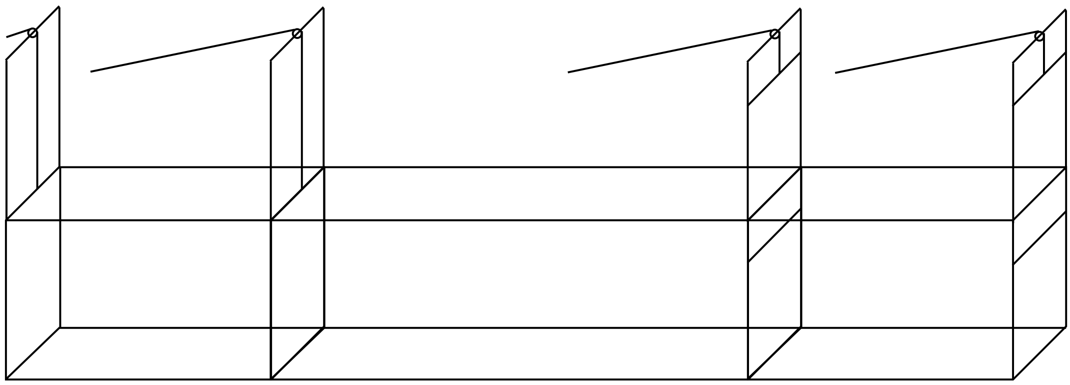

Figure 2. Crush cage

Table 1. African wild dogs involved in this study

Pack	Studbook ID	Gender	Age (Yr)	Genealogical relationship within the pack	Pack composition at study onset	Pack composition after regrouping
1	F322	♀	7	Dam of F423,F424,	Housed individually	Housed together
	F423	♀	5	Offspring of F322 Littermate of 424,	Housed individually	
	F424	♀	5	Offspring of F322 Littermate of F423,	Housed individually	
2	M484	♂	≥5*	Unknown*	Housed together	Housed together
	M488	♂	≥5*	Unknown*		
	M489	♂	≥5*	Unknown*		
	M485	♂	≥5*	Unknown*	Housed together	
	M487	♂	≥5*	Unknown*		
3	M273	♂	7	None	Housed together	Housed together
	M421	♂	5	Sibling of M518		
	M518	♂	1	none		
	M515	♂	1	Sibling of M421	Housed individually	
4	M368	♂	6	Littermate of F368	Housed together	Not applicable
	F364	♀	6	Littermate of F368 M368		
5	M213	♂	10	Sire of F403	Housed together	Not applicable
	F286	♀	8	Dam of F403		
	F403	♀	5	Offspring of M213 and F286		
6	M422	♂	5	None	Housed together	Not applicable
	M333	♂	7	None		

*Arrived at the Ann van Dyk Cheetah Centre - De Wildt together with 5 male and 2 female African wild dogs and functioned as a cohesive pack. Pack members were probably related. Males were split from the females. One male died in the single sex male group.

2.2 Experiment 1: DAP® perception

2.2.1 Experimental design

The objective of this experiment was to examine if African wild dogs perceive DAP®. The members of 4 wild dog packs (pack 1, 4, 5, 6; Table1) were exposed to areas treated with DAP® or placebo spray during separate trials. Pack 1 was first included in Experiment 2a & 2b and thereafter included in this experiment. During the first trial one area was treated with either DAP® or placebo spray. During a follow- up trial, after an interim period of 2 days, an equivalent area was treated with the other spray. The behavioural responses of pack members directed towards these different areas treated with different sprays were recorded and compared.

2.2.2 Behavioural observations

Each trial was performed according to the following protocol. The pack was confined to the corner camp during feeding (Fig. 1). Subsequently, an area (approximately 1m^2) in the adjacent main camp was treated by the observer with 30 ml of one of the two sprays. After application of the sprays animals were released into the main camp and the behavioural responses of the pack members directed toward the treated areas (Supplementary table 1) were recorded for 1 hour using continuous behaviour sampling and video recording (Martin and Bateson, 2007). The experiment was performed “blind". The DAP® and placebo spray were coded as Spray A and B by the supplier (Pherosynthese, Saint Saturnin les Apt, France) so the observer was not aware of the treatment during the experimental trials and data analysis.

2.2.3 Data- and statistical analysis

To examine if African wild dogs perceive DAP® acute behavioural responses directed towards the treated areas during the first 5 minutes after release were analysed and compared. In addition, the behavioural response directed towards the treated area during a period of 1 hour after release were analysed and compared. The frequency at which behavioural responses occurred was calculated for each subject from the observation data (real time observations and video observations were combined). To compare the behavioural responses directed towards the areas treated with the DAP and placebo spray, mean levels of behavioural responses were calculated for the DAP® and placebo trials and plotted for each pack. In addition, the proportion of the total observation time subjects spend resting within 1 adult dog length (ADL) of the treated area during each trial was calculated and compared. A Wilcoxon Signed Ranks Test was performed to test whether differences in mean levels behavioural responses directed towards treated with DAP® or placebo spray were statistically significant. The same statistical test was used to test whether the proportion of the total observation time subjects spend resting within 1 ADL of the treated area during both trials differed significantly. All tests were considered statistically significant at $P \leq 0.05$. Statistical analysis was performed with PASW Statistics 18 (IBM® SPSS® Statistics 18, SPSS Inc., IBM Company, Chicago, Illinois, USA)

2.3 Experiment 2a & 2b: Physiological validation of an EIA and the effect of DAP® on baseline stress levels

2.3.1 Experimental design

The experiment had two objectives. The first objective was to test whether changes in adrenocortical activity in African wild dogs, reflected by faecal GCM levels in the faeces, can be reliably monitored by using an cortisol-3-CMO EIA. An adrenal stimulation test (ACTH Challenge test) was performed to validate the EIA for monitoring faecal GCM levels and to demonstrate the physiological relevance of faecal GCMs as indicator of adrenocortical activity for African wild dogs. The second objective was to determine the effect of DAP® on baseline stress levels of captive African wild dogs. A non invasive faecal sampling technique was used to determine stress levels. Faecal GMC concentrations, which were used as indicator of stress, were quantified with a cortisol-3-CMO EIA.

The experiment was divided into five experimental phases: a habituation period, a baseline period, adrenal stimulation, a pre-treatment period and a DAP®-treatment period (Table 2). Faecal GMC levels, which proofed to be a reliable stress level indicator for several species (See for detailed reviews, Möstl and Palme, 2002; Palme et al., 2005; Touma and Palme, 2005), were monitored before and after adrenal stimulation and application of DAP® collars. The experiment was performed on three individually housed female African wild dogs (F322, F423, and F424) (Table 1, 2). Because individual response differences were expected for the adrenal stimulation and DAP®-treatment individual baseline measurements were made so that each study animal could serve as its own control.

Table 2. Experimental design and sample collection times

Start	Experimental phase	Treatment	Time of application (h)		Sample collection times (h) before / after ACTH administration
22-07-'09	Habituation period				
19-08-'09	Baseline period			F423	-20.25, -17.25, -15.50,
				F424	-20.00, -16.00,
				F322	-19.75, -15.25, -13.75,
21-08-'09	Adrenal stimulation	ACTH administration	6:45 am	F423	3.50, 8.25, 11.25, 32.00,
				F424	3.25, 8.50, 23.75,
				F322	3.00, 8.75
22-8-'09	Pre-treatment period			F423	35.00, 47.75, 54.75, 56.50, 58.75, 72.75, 80.25, 104.25,
				F424	33.00, 47.75, 56.50, 72.75, 81.75, 105.75,
				F322	11.25, 23.75, 30.75, 33.25, 47.75, 57.25, 72.75, 82.00, 106.00,
26-8-'09	DAP-treatment period	Immobilisation / Application DAP® collar	11.00 am	F423	168.25, 175.50, 179.25, 191.75, 198.75, 201.75, 216.00, 224.25, 226.75, 240.50
				F424	168.25, 175.50, 179.00, 191,75, 199.25, 202.75, 216.00, 224.25, 227.25, 240.50
				F322	168.25, 176.50, 191,75, 199.50, 202.25, 216.00, 224.50, 240.50

Habituation period

During the habituation period the three wild dogs were habituated to the experimental housing- and husbandry conditions, the experimental feeding regime and the faecal sample collection procedure. The already individually housed African wild dogs were captured and moved to three separate neighbouring enclosures 28 days before the start of the baseline period. For the duration of this experiment, each dog was held in the 2 corner camps of its own enclosure which were connected by a crush cage (Fig. 2).Throughout the day, the animals were allowed to move freely between the corner camps. Collection of fresh faecal samples was facilitated by confinement of the study animal to one of the corner camps by closing the sliding gates of the crush cage directly after defecation.

The normal feeding regime was adjusted by feeding the animals daily, between 6.45-7.00 AM, with a mixture of dry and moistened pellets from Eukanuba® only. For managerial reasons, the feeding time was incidentally changed. To determine the mean gut passage time for each animal food markers (dry rice or split peas) were added to the diet. The study animals were observed until they finished their food and after defecation fresh faeces was immediately checked for the presence of corresponding food markers.

Baseline period

During the baseline period, one day before the ACTH administration, fresh faecal samples were collected to provide a measure of baseline faecal GCM levels

Adrenal stimulation

For the adrenal stimulation test synthetic ACTH was administered to three African wild dogs. The animals were manually restrained using the crush cage in their camp (Fig. 2) and injected intramuscularly with 25 IU of synthetic ACTH (Synacthen®, Novartis RSA (Pty) Ltd, Kempton Park South-Africa) using a pole syringe. Synthetic ACTH was administered to each dog between 6.45 and 7.00 AM. The entire procedure took 5-10 min per wild dog. Fresh faecal samples were collected on the day of ACTH administration and the day after to monitor the rise and fall in faecal GMC levels as a result of the temporal adrenal stimulation by synthetic ACTH.

Pre-treatment period

During the pre-treatment period, which started the day after ACTH administration, fresh faecal samples were collected for a period of five days to provide a new measure of baseline faecal GCM levels in the three African wild dogs.

DAP® treatment period

To determine the effect of DAP® on stress levels DAP® collars were fitted to the neck of the three wild dogs for which immobilisation was necessary (Fig. 3). A combination of tiletamine-zolazepam (25-40 mg / animal) (Zoletil 100®, Virbac RSA (Pty) Ltd, Centurion, South Africa) and medetomidine HCl (0.7-1 mg per animal) (Domitor®, Pfizer Animal Health, Sandton, South Africa) was used to immobilise the animals. Study animals that could be manually restrained with a crush cage were injected intramuscularly using a pole syringe. The other animals were darted intramuscularly by means of a dart gun. To reduce the risks of regurgitation during induction, anaesthesia and recovery the animals were withheld from food ± 24 hr before and 24 hr after immobilisation Subsequent to the immobilisation procedure the subjects were provided with a DAP® collar. The length of the standard collars (70cm), which contain 2.5 % DAP®, was adjusted to the neck size of each dog (43-49 cm). The fitting was covered with adhesive tape to prevent early breaking. After application of the collar, the effects of medetomidine HCl were reversed with atipamizole HCl (2.5-3 mg / animal) (Antisedan®, Pfizer Animal Health, Sandton, South Africa). The dogs were immobilized and provided with a collar between 09.00 and 11.00 AM and the entire procedure took 15-30 min per wild dog.

Figure 3. An immobilized African wild dog provided with a DAP® collar.

2.3.2 Faecal sample collection

Faecal samples were collected during the baseline period, the adrenal stimulation test, the pre-treatment period and the DAP®-treatment period; ± 21 hrs before and ± 241 hrs after ACTH administration (Table 2; Supplementary table 2). Faecal samples could be collected 1 to 3 times a day. The moment of defecation could be roughly estimated based on earlier determined mean gut passage times. When the study animals were fed daily between 6.45-7.00 AM faecal samples could be collected 1 to 3 times a day. Added food markers could be found approximately 7, 10 and 24 hours after the time of feeding. During these periods of the day the animals were observed until they defecated. The sample collection times per animal are listed in Table 2. Fresh faecal samples were collected in plastic containers, frozen and stored at -20°C.

2.3.3 Faecal sample processing and extraction of steroids

The frozen faecal samples were transported on regular ice in an isolating box to the University of Pretoria. Transportation of the samples took approximately 1 hour. The vials with faeces were freeze dried using an Instruvac lyophilizer (Air & Vacuum Technologies, Midrand, South Africa) to control for water content. The freeze-dried faecal samples were homogenised with a strainer to control for fibre content. Approximately 50mg of faecal powder of each sample was extracted in 3 ml ethanol (80%) and vortexed for 15 min. on a multi-vortexer. After centrifugation at 2000 g for 10 min, the supernatants of the ethanolic suspensions were transferred to new tubes. The tubes with ethanol extracts of the faecal samples were stored at -20 °C until analysis with the EIA.

2.3.4 Determination of immunoreactivity using a cortisol-3-CMO EIA

The faecal ethanol extracts were analysed with a cortisol-3-CMO EIA (Palme and Möstl, 1997). Schatz and Palme (2001) found that this EIA detected the highest amount of immunoreactive metabolites in faecal samples of the domestic dog as compared with corticosterone- or 11-oxoetiocholanolone-EIAs. The antibody used for this EIA system was cortisol-3-CMO coupled with bovine serum albumin (BSA) and raised in rabbit. Biotinylated cortisol-3-CMO was used as label. The cortisol-3-CMO antibody cross-reacts with cortisol (4-pregnene-11β,17α,21-triol-3,20-dione) as standard 100%, corticosterone (4-pregnene-11β,21-diol- 3,20-dione) 6.2%, allodihydrocortisol (5α-pregnane-11β,17α,21-triol-3.20-dione) 4.6%, allotetrahydrocortisol (5α-pregnane-11β,17α,21-tetrol-20-one) 0.8% and tetrahydrocortisol 5β-pregnane-3α,11β,17α,21-tetrol-20-one (0.1%) (Palme and Möstl, 1997). The sensitivity of the EIA determined at 90% of maximum binding, was 1.5 pg/well. Inter- and intra-assay coefficients of variation ranged between 3.4% and 11.6%. The EIA was performed on microtiter plates according to the methods described earlier by (Palme and Möstl, 1997). Faecal hormone levels were expressed as ng/g dry weight (DW).

2.3.5 Data- and statistical analysis

Mean faecal GCM levels and standard deviations (SD) were calculated for the pre-treatment and DAP® treatment period. To assess the effect of DAP® on stress levels of the wild dogs the difference in mean faecal GCM levels for the pre-treatment and DAP® treatment period for each dog was calculated. However, the sample size was too low to achieve reliable statistical testing. To determine if faecal GCM levels decreased over time during the DAP®-treatment period Pearson's correlation coefficients between faecal GCM levels and time were calculated for the DAP® treatment period. As control period Pearson's correlation coefficients between faecal GCM levels and time were calculated for the pre-treatment period. All tests were considered statistically significant at $P \leq 0.05$. Statistical analysis was performed with PASW Statistics 18 (IBM® SPSS® Statistics 18, SPSS Inc., IBM Company, Chicago, Illinois, USA)

2.4 Experiment 3: The effect of DAP® on the behavioural process of regrouping

2.4.1 Experimental design

The objective of this experiment was to evaluate the effect of the application of DAP® on the behavioural process of regrouping. Members of 3 existing packs, which were separated because of severe aggression within the pack, were provided with a DAP® collar and treated with DAP® spray and subsequently regrouped. The behavioural process of regrouping was analysed by recording the social interactions and resting patterns of the African wild dogs several days after the regrouping.

2.4.2 Situation at study onset

All wild dogs involved in this experiment originated from 3 existing packs. At the onset of the study all wild dogs were housed individually or in small subgroups of 2 to 3 wild dogs (Table 1). The subgroups of pack 2 could already make contact through the fence because they were housed in adjacent camps. The same is true for the subgroups of pack 3. For members of pack 1, on the other hand, this was not possible because they were individually housed in separate surrounding enclosures for the purpose of experiment 2.

Figure 4. Application of DAP® spray through the air holes of the crates

2.4.3 Regrouping procedures

Application of DAP® collars and DAP® spray

DAP® collars were fitted to the neck of the wild dogs approximately 1 week before the regrouping event. For this purpose wild dogs were immobilized according to the methods earlier describe in the section of experiment 2 (Fig. 3). DAP® spray was directly applied on the flanks of African wild dogs on the day of regrouping (Fig. 4)

Regrouping of Pack 1

On the day of the regrouping event two (F424, F322) of the three wild dogs were crated. Prior to the crating all sides of the crates were treated with DAP® spray (± 30ml). 15-20 min before the release DAP® spray (± 30ml) was directly applied on the flanks of the wild dog through the air holes of the crates. The third dog (F423) was not crated because this wild dog stayed in a corner camp adjacent to the enclosure where the dogs were released. DAP® spray □± 60 ml□ was therefore directly sprayed on the flank of the wild dog in the crush cage. For the release the two crates were placed next to each in the main camp of the enclosure. The wild dog in the corner camp was released first into the main camp and immediately thereafter the two crated dogs were released from their crates at other end of the main camp.

Regrouping of Pack 2

On the day of the regrouping event all 5 wild dogs were crated. Prior to the crating all sides of the crates were treated with DAP® spray (± 30ml). 20-30 minutes before the release DAP® spray was directly applied on the flanks of the wild dog flanks through the air holes of the crates. The crates were placed next to each other and all dogs were released at once in the same direction into the main camp of the enclosure.

Regrouping of Pack 3

On the day of the regrouping event all 4 wild dogs were crated. Prior to the crating all sides of the crates were treated with DAP® spray (± 30ml). 2 hours before the release DAP® spray was directly applied on the flanks of the wild dog flanks through the air holes of the crates. The crates were placed next to each other and all dogs were released at once in the same direction into the main camp of the enclosure.

2.4.4. Behavioural observations

Free living African wild dogs are socially active in the early morning and late afternoon and typically rest for the after the morning hunt. During this resting period pack members are divided in one or more subgroups. The resting pattern of African wild dogs reflects the relative strength of social bonds between pack members and the degree of social integration within the wild dog pack (de Villiers et al., 2003; Gusset et al., 2006; Knobel and Du Toit, 2003; McCreery, 2000)

To evaluate the effect of DAP® collars and DAP® sprays, the behavioural process of regrouping was analysed by recording social interactions and resting patterns (Fig. 5). Behavioural data was collected the first 7 days and 9th and 13th day after pack formation. Behavioural observations were made through out the day including both active periods (early morning and late afternoon) and resting periods resulting in a total observation time of 53 hr for pack 1, 38 hr for pack 2 and 49 hr for pack 3. Mean observation time per day for pack 1 5,89 hr (min. 2 hr; max. 9 hr), 4,75 hr (min. 1,5 hr; max. 6,75 hr) for pack 2 and 5,44 hr (min. 4hr, max. 7,25) for pack 3. The wild dogs were identified based on variation in coat colouring and distinctive marks such as torn ears, damaged tails, scars or stiff limbs.

Figure 5. (A) An African wild dog (left) elicits submission from another wild dog (middle) by approaching in a dominant manner using a stalk approach (see supplementary table 3). (B) The receiving wild dog (right) responds towards the actor of the stalk approach in a submissive manner by showing active submission (see supplementary table 3).

To determine at which level social interactions (*e.g.* aggressive and affiliative interactions) occur during regrouping and to identify changes in social interactions over time, hourly rates of social behaviour were recorded using continuous behaviour sampling (Martin and Bateson, 2007). Social interactions were classified into 6 major categories: severe aggressive behaviour, ritualized / stylised aggressive behaviour, dominant behaviour, submissive behaviour, social play and affiliative behaviour, partially based on the categorisation of (Sands and Creel, 2004) (Supplementary table 3). The category “Severe (uninhibited) aggressive behaviour /attack’’, was reserved for escalated / vigorous fighting associated with high levels of agonistic arousal and characterised by uninhibited biting in which it appeared that injury was a real possibility. The category “Ritualized / stylised / aggressive behaviour’’, was reserved for ritualized (inhibited) aggressive behaviour associated with low levels of agonistic arousal and characterised by inhibited biting in which it appeared that there was a relatively low risk for injury. The category “Dominant behaviour’’ included any agonistic (threat) displays, gestures and assertive agonistic behaviour related to dominance that did not involve physical contact. The category “Submissive behaviour’’ included displays, gestures and behaviour indicating that an individual surrenders, submits and not retaliate as a response on an attack or threat by an opponent or by actively seeking contact with the recipient. The category “social play” refers to playful non-competitive fighting in which attacker and defender exchanges roles and no winner or loser emerges and interactions rarely include behaviours which can inflict injury. The category “Affiliative behaviour” included friendly, positive and appeasing displays, gestures and behaviour which promotes the social bond between individuals or / and enhances group cohesion. For a detailed description of the behaviours within these behavioural categories see Supplementary table 3 based on Derix (1994) and Fox (1969; 1971)

To evaluate the relative strength of social bonds between pack members and the degree of social integration within the wild dog pack resting associations between members of the regrouped packs were recorded throughout the day. In a prior pilot study on a pack of captive wild dogs no clear / strict resting period was found. Pack members resting within two ADLs of another were recorded as being in one subgroup following McCreery (2000). Wild dogs resting alone, more than 2 ADLs from other pack members, were recorded as being in separate subgroups. Resting associations were recorded after each disturbance that led to disruption of the resting pattern, to avoid autocorrelation of the data. Observations of resting associations were only included in the data analysis when all pack members were resting and the resting pattern was stable for ≥ 10 min. An index of resting association was determined by calculating a simple ratio association index (AI, Cairns and Schwager, 1987) between individual A and B as $AI_{AB} = X/(X+Y))$, where X is the number of resting bouts during which A and B are observed together in the same subgroup and Y is the number of resting bouts during which A and B are observed in separate subgroups. Based on resting association indices a single-link cluster analysis (SLCA) dendrogram was constructed (Morgan et al., 1976).

2.4.5 Data- and statistical analysis

Hourly rates of severe aggression, ritualized aggression, dominant behaviour, affiliative behaviour and social play were calculated for each observation day. Frequencies of these categories of social behaviour were plotted against time separately to determine daily rates of occurrence and to identify changes in these social interactions over time. Social interactions that occurred in the context of food competition were analysed separately using similar methods. Indices of resting associations were calculated for every combination of pack members and for each observation day to determine changes in the strength of social bonds between pack members over time. To determine if occurrence frequencies of severe aggression, ritualized aggression, dominant behaviour, affiliative behaviour and social play changed over time during the two weeks after regrouping Pearson’s correlation coefficients between occurrence frequencies and time were calculated. To determine if the relative strength of resting associations between pack members changed over time during the two weeks after regrouping Pearson’s correlation coefficients between occurrence frequencies and time were calculated. All tests were considered statistically significant at $P \leq 0.05$. Statistical analysis was performed with PASW Statistics 18 (IBM® SPSS® Statistics 18, SPSS Inc., IBM Company, Chicago, Illinois, USA)

3. Results

3.1 Experiment 1: DAP® perception

3.1.1 Behavioural response during the first 5 minutes after release

To examine if African wild dogs perceive DAP® acute behavioural responses directed towards areas treated with DAP® or placebo spray during the first 5 minutes after release were analysed and compared. Figure 6 shows mean frequencies of behavioural responses directed towards the treated areas for each pack during the first 5 minutes.

Sniffing

During the DAP® trial a higher mean level of sniffing within 1 ADL of the treated area was observed for pack 1 and 4. No differences were found between the DAP® and placebo trial for pack 5 and 6. Occurrence frequencies of sniffing behaviour did not differ significantly between the DAP® and placebo trial for the four tested packs (Wilcoxon Signed Ranks Test: Z=-0,816; P=0,414). However, in two of the four packs, higher occurrence frequencies of sniffing behaviour within 1 ADL of the treated area were observed during the DAP® trial.

Resting

During the first 5 minutes of the DAP® and placebo no resting behaviour within 1 ADL of the treated area was observed for the four packs.

Urinating

During the DAP® trial a higher mean level of urinating within 1 ADL of the treated area was observed for pack 4 and pack 5. No differences were found between the DAP® and placebo trial for pack 1 and 6. Occurrence frequencies of urinating did not differ significantly between the DAP® and placebo trial for the four tested packs (Wilcoxon Signed Ranks Test: Z=-1,342; P=0,180). However, in two of the four packs, higher occurrence frequencies of urinating within 1 ADL of the treated area were observed during the DAP® trial.

Licking mandibular area

During the DAP® trial a higher mean level of licking the mandibular region within 1 ADL of the treated area was observed for pack 4. No differences were found between the DAP® and placebo trial for pack 1, 5 and 6. Occurrence frequencies of licking the mandibular region did not differ significantly between the DAP® and Placebo trial for the four tested packs (Wilcoxon Signed Ranks Test: Z=-1,000; P=0,317). However, in one of the four packs, a higher occurrence frequency of licking the mandibular region within 1 ADL of the treated area was observed during the DAP® trial.

Panting

During the DAP® trial a higher mean level of panting within 1 ADL of the treated area was observed for pack 4 and 5. No differences were found between the DAP® and placebo trial for pack 1 and 6. Occurrence frequencies of panting did not differ significantly between the DAP® and placebo trial for the four tested packs (Wilcoxon Signed Ranks Test: Z=-1,342; P=0,180). However, in two of the four packs, higher occurrence frequencies of panting within 1 ADL of the treated area were observed during the DAP® trial.

Sum of all categories

To determine if the wild dogs packs in general responded more to areas treated with DAP® or placebo spray the different evaluated categories of behaviour were combined into a single category "behaviour directed towards the treated area". For pack 4 and 5 a higher mean level of behaviour directed towards the treated area was observed during the DAP® trial. No differences were found between the DAP® and placebo trial for pack 1 and 6 (Wilcoxon Signed Ranks Test: Z=-1,604; P=0,109). However, in two of the four packs, in general higher occurrence frequencies of "'behaviour directed towards the treated area" were observed during the DAP® trial.

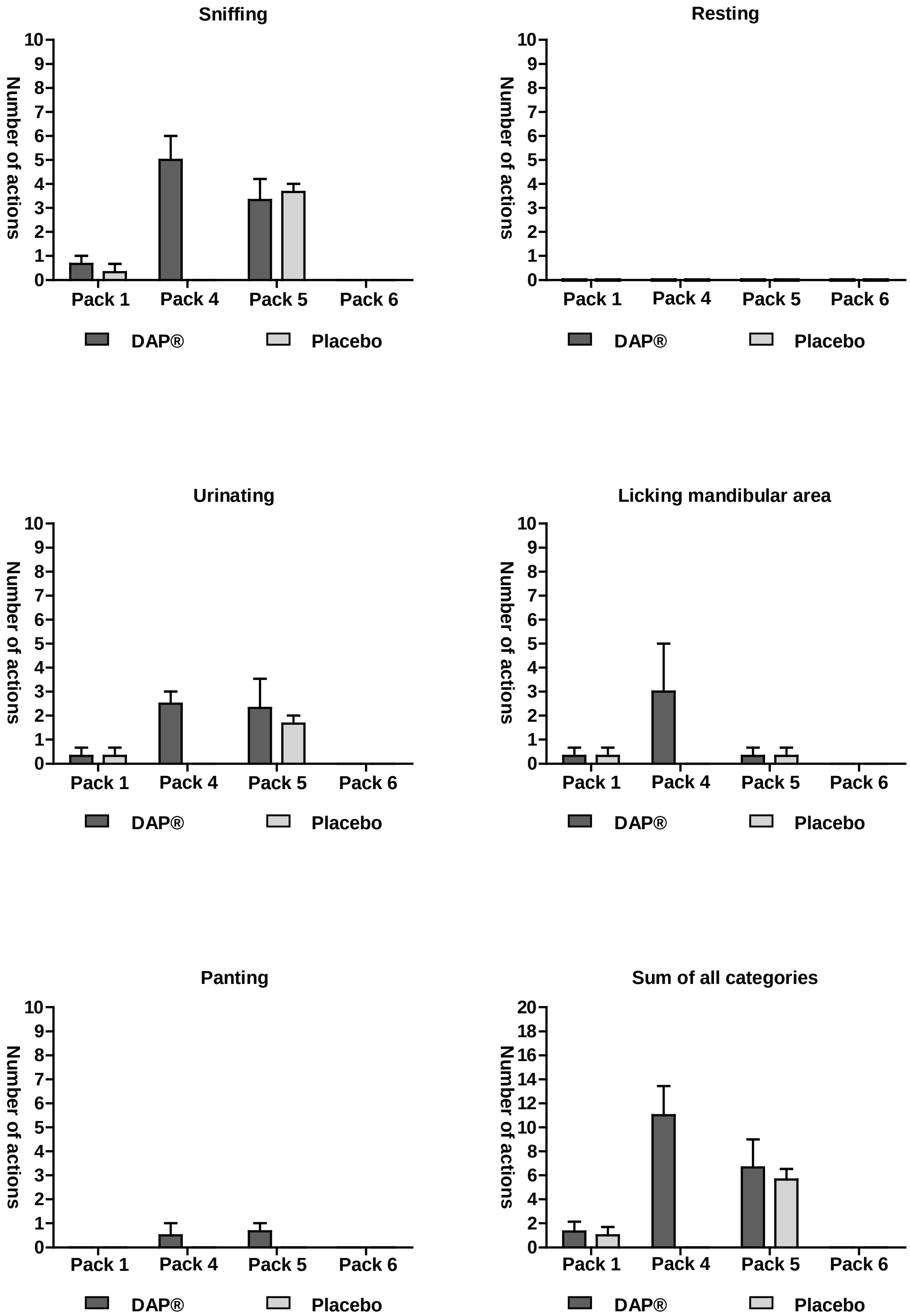

Figure 6. Bars indicate the mean occurrence of behavioural responses directed towards areas treated with DAP® or placebo during the first 5 minutes after release. Error bars indicate standard deviations

3.1.2 Behavioural response during a 1 hour observation period after release

Furthermore behavioural responses directed towards areas treated with DAP® or placebo during a period of 1 hour after release were analysed and compared. Figure 7 shows mean frequencies of behavioural responses directed towards the treated areas for each pack during a 1 hour observation period.

Sniffing

During the DAP® trial a higher mean level of sniffing within 1 ADL of the treated area was observed for pack 4. For pack 5 and 6, on the other hand, higher mean levels of sniffing within 1 ADL of the treated area were observed during the placebo trial. No differences were found between the DAP® and placebo trial for pack 1. Occurrence frequencies of sniffing behaviour did not differ significantly between the DAP® and placebo trial for the four tested packs (Wilcoxon Signed Ranks Test: Z=0,000; P=1,000). In two of the four packs, higher occurrence frequencies of sniffing behaviour within 1 ADL of the treated area were observed during the placebo trial. While in a third pack higher occurrence frequencies of sniffing behaviour within 1 ADL of the treated area were observed during the DAP® trial.

Resting

During the DAP® trial a higher mean level of resting behaviour within 1 ADL of the treated area was observed for pack 4. For pack 1, 5 and 6 no differences were found between the DAP® and placebo trial. Occurrence frequencies of resting behaviour did not differ significantly between the DAP® and placebo trial for the four tested packs (Wilcoxon Signed Ranks Test: Z=-1,000; P=0,317). However, in one of the four packs, higher occurrence frequencies of resting behaviour within 1 ADL of the treated area were observed during the DAP® trial.

Urinating

During the DAP® trial a higher mean level of urinating within 1 ADL of the treated area was observed for pack 1, 4 and pack 5. For pack 6, on the other hand, a higher mean level of urinating within 1 ADL of the treated area was observed during the placebo trial. Occurrence frequencies of urinating did not differ significantly between the DAP® and placebo trial for the four tested packs (Wilcoxon Signed Ranks Test: H=-1,095; P=0,273). However, in three of the four packs, higher occurrence frequencies of urinating behaviour within 1 ADL of the treated area were observed during the DAP® trial. While in another pack higher occurrence frequencies of urinating behaviour within 1 ADL of the treated area were observed during the placebo trial.

Licking mandibular area

During the DAP® trial a higher mean level of licking the mandibular region within 1 ADL of the treated area was observed for pack 1 and 4. For pack 5, on the other hand, higher mean level of licking the mandibular region within 1 ADL of the treated area was observed during the placebo trial. For pack 6 no differences were found between the DAP® and placebo trial. Occurrence frequencies of licking the mandibular region did not differ significantly between the DAP® and placebo trial for the four tested packs (Wilcoxon Signed Ranks Test: Z=-0,535; P=0,593). However, in two of the four packs, higher occurrence frequencies of licking the mandibular region within 1 ADL of the treated area were observed during the DAP® trial. While in another pack higher occurrence frequencies of panting within 1 ADL of the treated area were observed during the placebo trial.

Panting

During the DAP® trial a higher mean levels of panting within 1 ADL of the treated area were observed for pack 1, 4 and pack 5. For pack 6 no differences were found between the DAP® and placebo trial. Occurrence frequencies of panting did not differ significantly between the DAP® and placebo trial for the four tested packs (Wilcoxon Signed Ranks Test: Z=-1,604; P=0,109). However, in three of the four packs, higher occurrence frequencies of panting within 1 ADL of the treated area were observed during the DAP® trial.

Sum of all categories

To determine if the wild dogs packs in general responded more to areas treated with DAP® or placebo spray the evaluated categories of behaviour were combined into a single category "behaviour directed towards the treated area" . For pack 1 and 4 higher mean level of behaviour directed towards the treated area was observed during the DAP® trial. For pack 6 a higher mean level of behaviour directed towards the treated area was observed during the placebo trial. For pack 5 no differences was found between the DAP® and placebo trial. Mean levels of behaviour directed towards the treated area did not differ significantly between the DAP® and placebo trial for the four tested packs (Wilcoxon Signed Ranks Test: Z=-0,365, P=0,715). However, in two of the four packs, in general higher occurrence frequencies of "'behaviour directed towards the treated area" were observed during the DAP® trial. While in a third pack, in general higher occurrence frequencies of "'behaviour directed towards the treated area" were observed during the placebo trial.

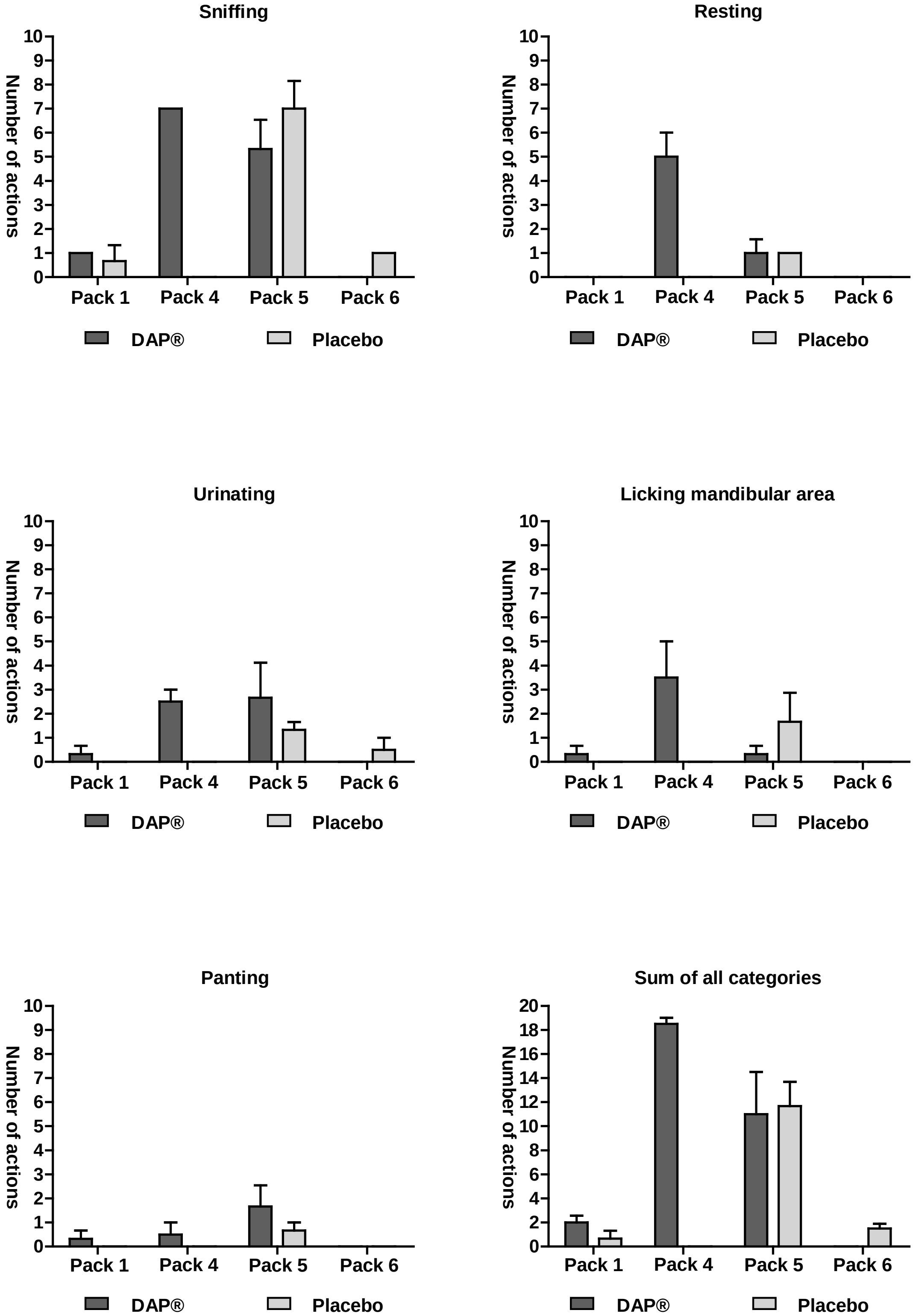

Figure 7. Bars indicate the mean occurrence of behavioural responses directed towards areas treated with DAP® or placebo spray during an observation period of 1 hour after release. Error bars indicate standard deviations

3.1.3 Resting behaviour during a 1 hour observation period after release

In addition, the proportion of the total observation time subjects spend resting within 1 ADL of the treated area during the DAP® and placebo trial was calculated and compared. Figure 8 shows the mean amount of observation time pack members spent on resting within 1 ADL of the pack members. During the DAP® trial members of pack 4 and 5 spent on average more time on resting behaviour within 1 ADL of the treated area than during the placebo trial. Members of pack 1 and pack 6 showed no resting behaviour within 1 ADL of the treated areas. The mean amount of observation time pack members spent on resting within 1 ADL of the treated area did not differ significantly between the DAP® and placebo trial for the four tested packs (Wilcoxon Signed Ranks Test: =-1,342; P=0,180). However, in two of the four packs pack members spent more time on resting within 1 ADL of the area treated with DAP®.

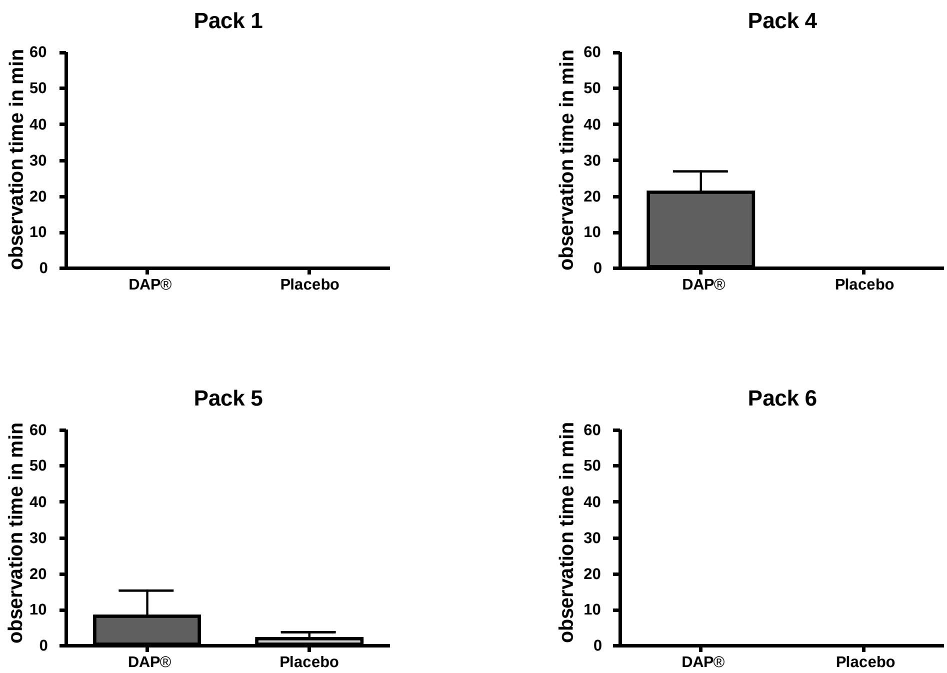

Figure 8. Bars indicate the proportion of total observation time subjects spend resting in area treated with DAP® or placebo spray during an observation period of 1 hour. Error bars indicate standard deviations

3.2 Experiment 2a & 2b: Physiological validation of an EIA and the effect of DAP® on baseline stress levels

3.2.1 Gut passage times

When the study animals were fed once a day between 6.45-7.00 AM, faecal samples could be collected 1 to 3 times a day depending on faecal production. Food markers were added once daily and could be found approximately 8, 11 and 24 hours after time of feeding (Table 3).

Table 3. The minimum, maximum and mean gut passage time in three African wild dogs

			Gut passage time				
Subject	Feeding time[a]	Collection period	Min[b]	Max[b]	Mean[b]	*n*[c]	SD
F423	6.45-7.00 AM	1	6,75	8,50	7,25	9	0,66
		2	10,00	11,50	10,83	5	0,49
		3	23,75	27,50	24,59	8	1,22
F424	6.45-7.00 AM	1	7,50	9,00	8,22	8	0,57
		2	9,75	11,25	10,69	4	0,66
		3	23,50	27,25	24,42	9	1,12
F322	6.45-7.00 AM	1	6,75	9,25	8,53	9	0,82
		2	10	11	10,44	4	0,43
		3	23,50	27,00	24,47	9	1,02

[a] For managerial reasons, the feeding time was incidentally changed
[b] Minimum, maximum and mean amount of time after which faecal samples could be collected
[c] Number of faecal samples

3.2.2 Physiological validation of a cortisol-3-CMO EIA

As a result of adrenal stimulation a rise and fall in faecal GCM levels in the three study animals was observed (Fig. 5a, c and e). Faecal GCM levels of the three study animals raised up to 268, 256%, and 190% compared to the mean faecal GCM levels of the baseline period. The minimum, maximum and mean baseline faecal GCM levels and the absolute and relative peak faecal GCM levels are given in Table 4. Peak faecal GCM levels were reached 8,25-8,75 hours after ACTH administration. 11,25-35 hours after ACTH administration faecal GCM levels returned within the range of baseline levels (Fig. 5 a, c, e).

Table 4. The minimum, maximum and mean faecal GCM levels and absolute and relative peak levels measured during the baseline period and after adrenal stimulation in three African wild dogs

	Baseline period					Adrenal stimulation		
Subject	Min[a]	Max[a]	Mean[a]	*n*[b]	SD	Absolute peak faecal GCM level[a]	Relative peak faecal GCM level (%)[c]	Delay of faecal GCM excretion (h)[d]
F423	88,92	147,50	119,33	3	23,97	320,35	268	8,25
F424	98,18	156,05	127,12	2	28,93	325,43	256	8,50
F322	74,90	109,25	93,80	3	14,23	178,28	190	8,75

[a] Faecal GCM levels expressed in ng/g DW faeces
[b] Number of faecal samples
[c] Peak faecal GCM levels expressed relative to the corresponding mean baseline faecal GCM levels
[d] Minimum, maximum and mean amount of time after which peak faecal GCM levels were observed in the faeces subsequent to ACTH administration

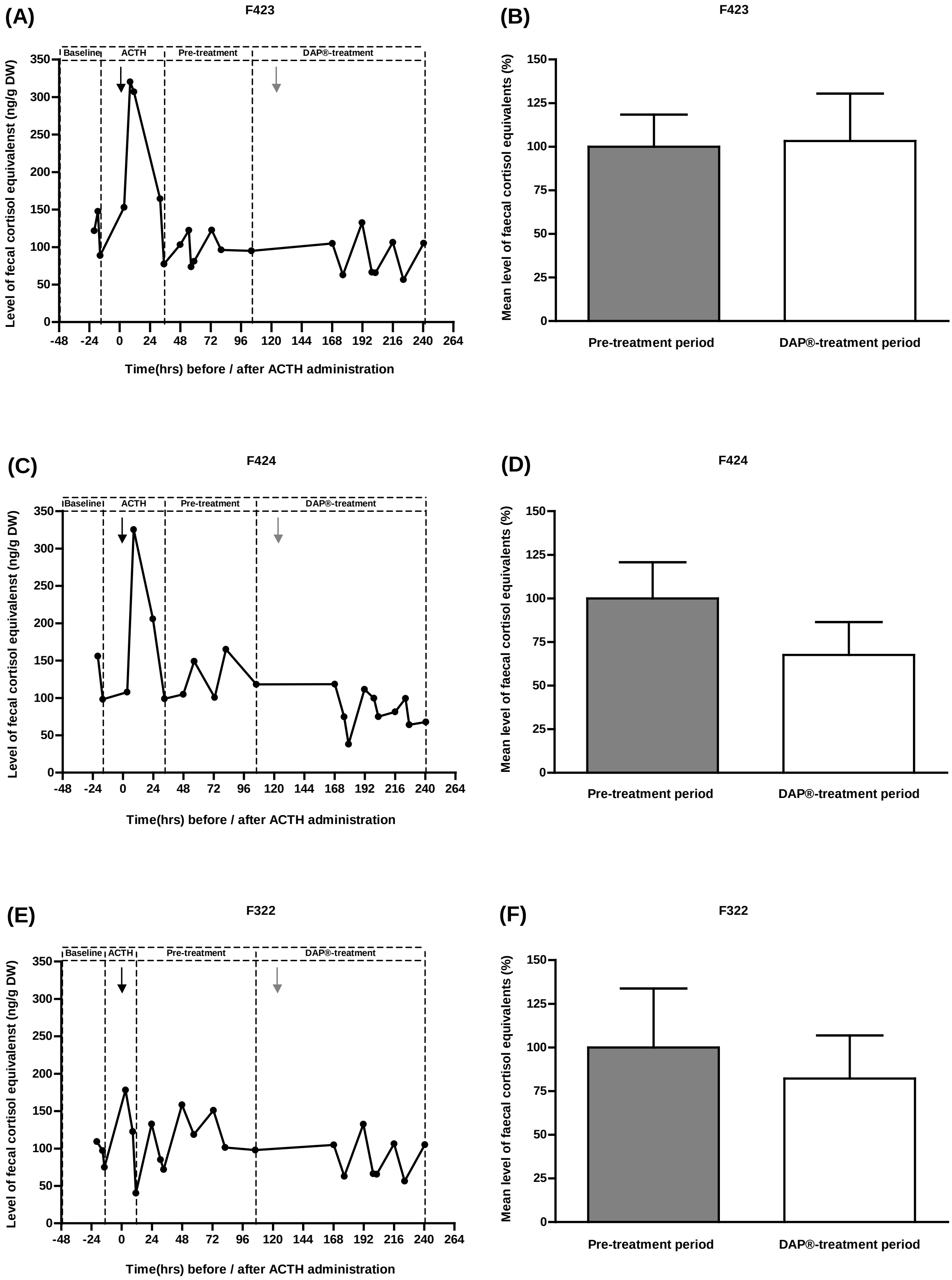

Figure 9. Graph A, C and E show faecal GCM concentrations of three female African wild dogs during a baseline period, an adrenal stimulation test, a pre-treatment period and a DAP®-treatment period. *Black arrows* indicate time of ACTH administration. *Grey arrows* indicate time of application of DAP® collars. Graph B, D and F show mean faecal GCM concentrations of the three female African wild dogs during the pre-treatment and the DAP®-treatment period. Error bars indicate standard deviations.

3.2.3 The effect of DAP® on baseline stress levels

To assess if exposure to DAP® decreased stress levels of the wild dogs during the treatment period, mean faecal GCM levels of the pre-treatment period were compared with mean faecal GCM levels of the DAP®-treatment period. In one dog no substantial difference in mean faecal GCM levels (103%) was observed during the DAP® exposure period relative to the pre-treatment period (Fig. 5b). In the other animals considerable lower mean faecal GCM levels (68% and 82%) were observed during the DAP® exposure period relative to the pre-treatment period (Fig. 5d and f). The minimum, maximum and mean faecal GCM levels of the pre-treatment and DAP®-treatment period and the relative increase / decrease of mean faecal GCM levels during the DAP®-treatment period are given in Table 5.

Table 5. The minimum, maximum and mean faecal GCM levels during the of the pre-treatment and DAP®-treatment period and the relative increase / decrease of mean faecal GCM levels (%) during the DAP®-treatment period compared to the pre-treatment period for three African wild dogs period.

	Pre-treatment period					DAP®-treatment period					Relative increase / decrease of mean faecal GCM levels (%)
Subject	Min[a]	Max[a]	Mean[a]	*n*[b]	SD	Min[a]	Max[a]	Mean[a]	*n*[b]	SD	
F423	73,50	122,58	96,41	8	18,43	58,81	204,29	99,55	10	27,18	+3%
F424	98,95	165,23	122,84	6	20,71	38,21	118,47	83,01	10	18,84	-32%
F322	40.44	158,52	106,41	9	33,80	56,46	132, 47	87,52	8	24,62	-18%

[a] Faecal GCM levels expressed in ng/g DW faeces
[b] Number of faecal samples
[c] The increase decrease of mean faecal GCM levels during the DAP treatment period expressed in relative to the corresponding mean faecal GCM levels of the pre-treatment period

During the pre-treatment period faecal GCM levels of the three study animals rose to some extent over time (Fig 9. a, c and e). Weak positive correlations between faecal GCM levels and time were found in all three study animals although not significant.
In contracts, faecal GCM levels of the three study animals decreased to some extent over time during the DAP®-treatment (Fig 9. a, c and e). For two of the three study animals weak negative correlations between faecal GCM levels and time during the DAP®-treatment were found although not significant. Pearson's correlation coefficients and p-values are shown in Table 6.

Table 6. Correlation between faecal GCM levels over time

	Pre-treatment period			DAP®-treatment period		
Subject	***n***[a]	**Pearson´s *r***[b]	**P-value**[b]	***n***[a]	**Pearson´s *r***[b]	**P-value**[b]
F322	9	0,323	0,184	8	-0,027	0,475
F423	8	0,218	0,302	10	-0,412	0,119
F424	6	0,333	0,260	10	-0,189	0,300

[a] Number of faecal samples
[b] One-tailed

3.3 Experiment 3: Effect of DAP® on the behavioural process of regrouping

3.3.1 Social interactions

Hourly rates of 5 categories of social behaviour within the pack are shown in Figure 10.

Pack 1

Severe aggressive behaviour was only observed on the day of regrouping. Ritualized aggressive behaviour was observed on the 2nd and the 4th day. Social play behaviour was not observed within this pack. The occurrence frequency for these three behavioural categories was too low to test whether frequency changed significantly over time. Dominant and affiliative behaviour was observed during the entire study period. However, no significant changes in frequencies of dominant behaviour (ρ=-0,083; P=0,831/>0,05; n=9) and affiliative behaviour (ρ=0,167,P=0,668/>0,05, n=9) were observed overtime (Fig. 10).

Pack 2

Severe aggressive and ritualized aggressive behaviour were only observed on the day of regrouping. Social play behaviour was not observed within this pack. The occurrence frequency for these three behavioural categories was too low to test whether frequency changed significantly over time. A high level of dominant behaviour was observed within the pack during the 1st day. However, dominant behaviour declined significantly overtime (ρ=0,039/<0,05; n=8). Affiliative behaviour was observed during the entire study period but the occurrence frequency did not change significantly (ρ=-0,143; P=0,736/ >0,05; n=8) overtime (Fig. 10).

Pack 3

Severe aggressive behaviour was only observed on the 9th day of regrouping. A high level of dominant behaviour and play behaviour was observed within the pack during the 1st day and lower levels were observed overtime. Ritualized aggressive behaviour and affiliative behaviour were observed during the entire study period Changes in the occurrence frequency of dominant behaviour (ρ=-0,460; P=0,213 />0,05; n=9), play behaviour (ρ=-0,619; P=0,075 />0,05; n=9) and ritualized aggression (ρ=-0,467; P=0,205 />0,05; n=9) overtime were not significant. Affiliative behaviour declined significantly (ρ=-0,700; P=0,036 / <0,05; n=9) overtime (Fig. 10).

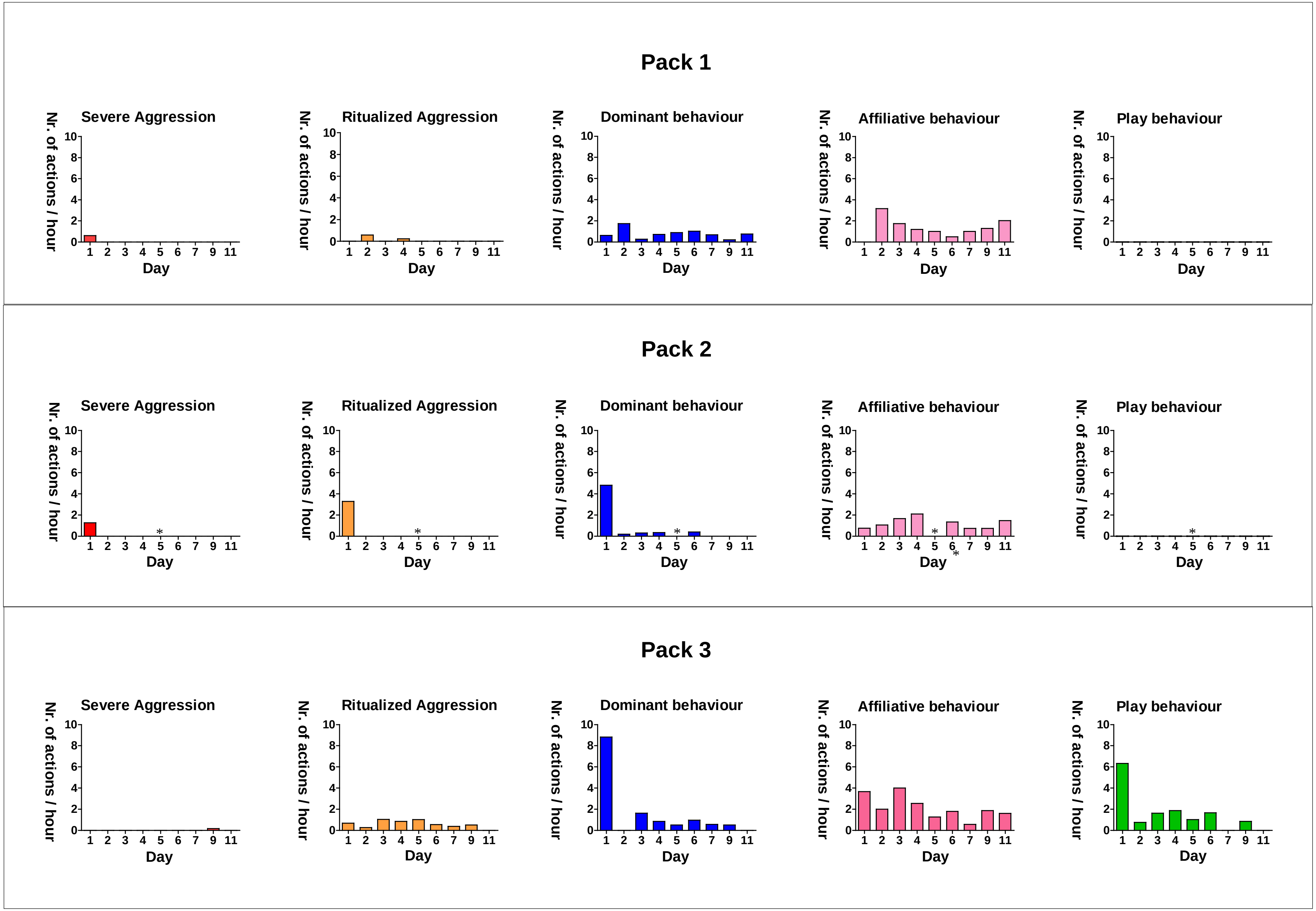

Figure 10. Frequencies of five categories of social behaviour shown by the pack members of group 1, 2 and 3 during the first eleven days after the introduction event. * No behavioural observations were made.

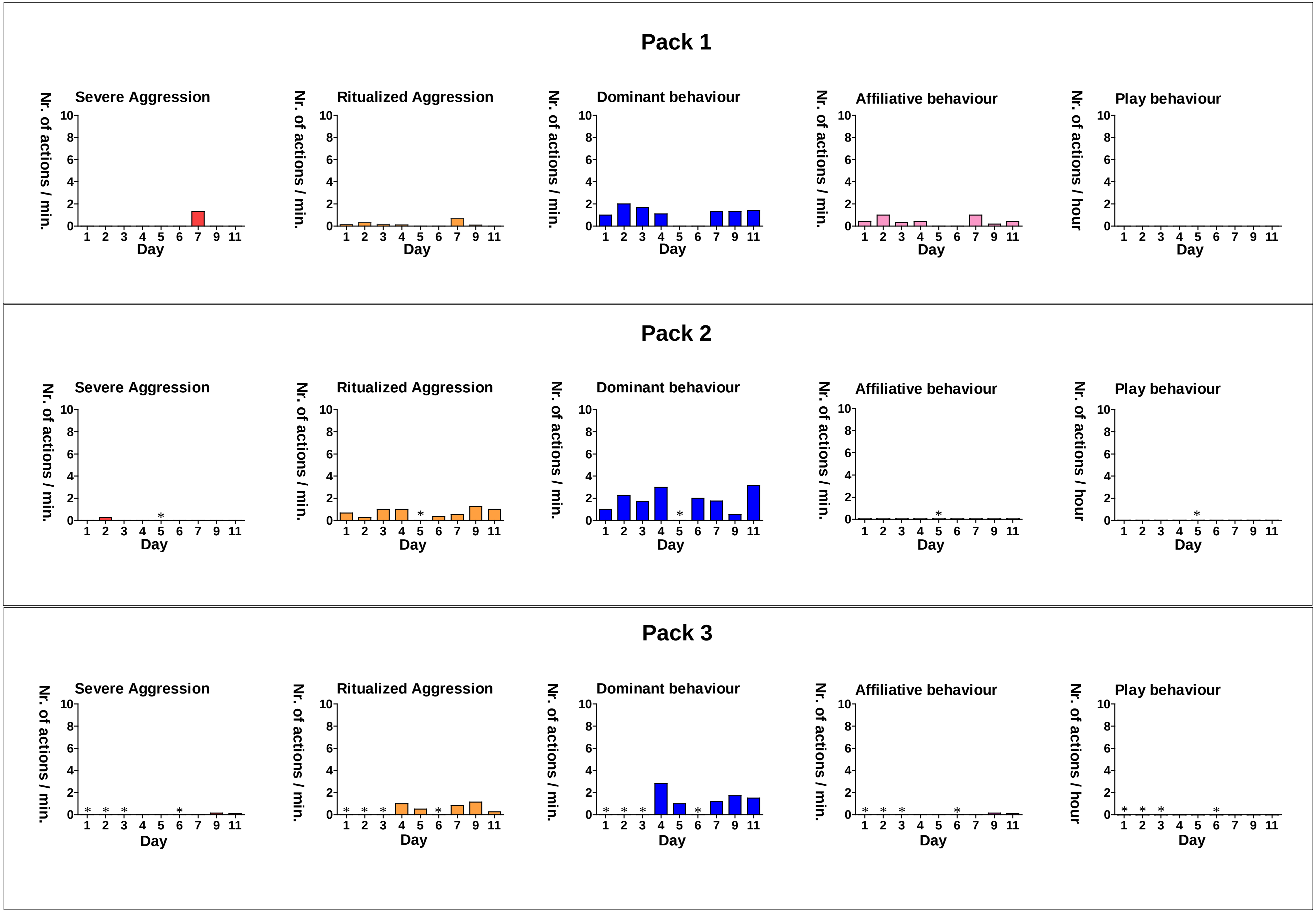

Figure 11. Frequencies of five categories of social behaviour in the context of food shown by the pack members of group 1, 2 and 3 during the first eleven days after the introduction event. * No behavioural observations were made.

Hourly rates of 5 categories of social behaviour within the pack in context of food competition are shown in Figure 11.

Pack 1

Severe aggressive behaviour in the in context of food competition was only observed on the 7th day after regrouping. Social play behaviour in the in context of food competition was not observed within this pack. The occurrence frequency for these two behavioural categories was to low to test whether frequency changed significantly over time. Compared to normal frequencies (Acts/hr) of ritualized aggression and dominant (Fig. 10) frequencies (Acts/min) of these behavioural categories in the in context of food competition were relative high. No significant changes in frequencies of ritualized aggression ($\rho=0,439$; $P=0,276$ / $>0,05$; $n=9$), dominant behaviour ($\rho=-0,012$; $P=0,978/>0,05$; $n=8$) and affiliative behaviour ($\rho=-0,337$, $P=0,414/>0,05$, $n=8$) in the in context of food competition were observed overtime (Fig. 11).

Pack 2

Severe aggressive was only observed on the 2nd day after regrouping. Social play behaviour and affiliative behaviour in the in context of food competition were not observed within this pack. The occurrence frequency for these three behavioural categories was too low to test whether frequency changed significantly over time. Compared to normal frequencies (Acts/hr) of ritualized aggression and dominant (Fig. 10) frequencies (Acts/min) of these behavioural categories in the in context of food competition were relative high. No significant changes in frequencies of ritualized aggression ($\rho=0,439$; $P=0,276$ / $>0,05$; $n=8$) and dominant behaviour ($\rho=0,214$; $P=0,610$ / $>0,05$; $n=8$) in the in context of food competition were observed overtime (Fig. 11).

Pack 3

Severe aggressive behaviour and affiliative behaviour was only observed on the 9th and 11th day after regrouping. Social play behaviour in the in context of food competition was not observed within this pack. The occurrence frequency for these three behavioural categories was too low to test whether frequency changed significantly over time. Compared to normal frequencies (Acts/hr) of ritualized aggression and dominant behaviour (Fig. 10) frequencies (Acts/min) of these behavioural categories in the in context of food competition were relative high. No significant changes in frequencies of ritualized aggression ($\rho=-0,300$; $P=0,624$ / $>0,05$; $n=8$) and dominant behaviour ($\rho=-0,100$; $P=0,873$ / $>0,05$; $n=8$) in the in context of food competition were observed overtime (Fig. 11).

3.3.2 Resting associations

The single-link cluster analysis dendrogram based on resting association indices shows that F423 and F424 of pack 1 are paired into one subgroup (Fig. 12). This corresponds with the genealogical relationship within the pack, F423 and F424 are littermates. F423 and F424 rested less frequent in association with F322, the dam of F423 and F424.

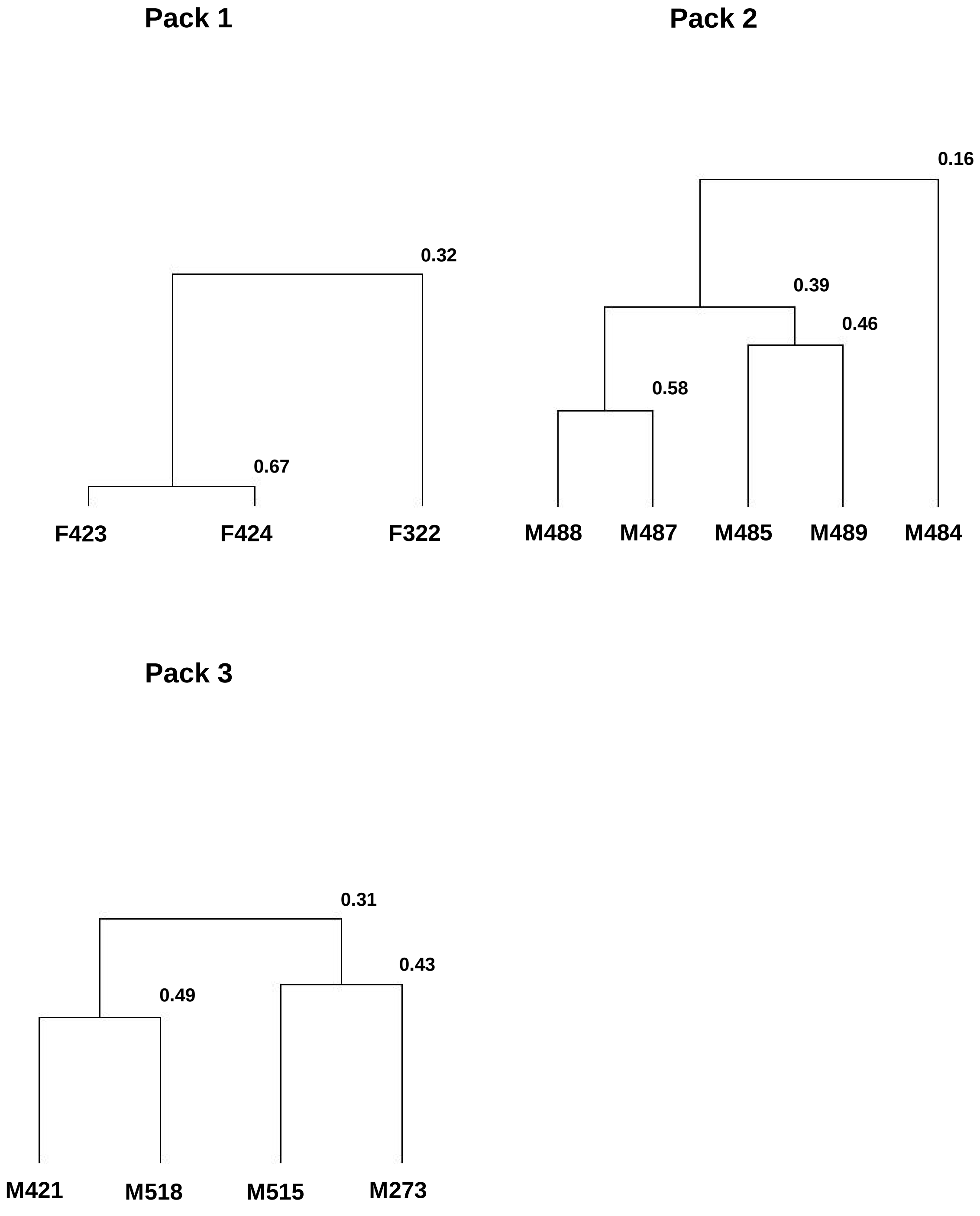

Figure 12. Single-link cluster analysis dendrograms of resting association patterns between members of pack 1, 2 and 3. Indices of association between pairs or subgroups of individuals are indicated on the dendrograms.

Table 7. Resting association indices between members of pack 1, 2 and 3 during the first 7 days, the 9th and 11th day after regrouping

Group 1												
	Day										Correlation over time	
Resting association	1	2	3	4	5	6	7	9	11	Mean	Pearson´s r^b	P-value^b
F423-F424	~	0,38	1,00	0,33	0,60	~	0,86	0,50	1,00	0,67	0,415	0,307
F423-F322	~	0,25	0,11	0,08	0,20	~	0,57	0,00	1,00	0,32	0,311	0,453
F424-F322	~	0,25	0,11	0,50	0,20	~	0,71	0,00	1,00	0,40	0,359	0,382
Mean	~	0,29	0,41	0,31	0,33	~	0,71	0,17	1,00		0,407	0,317
Group 2												
	Day										Correlation over time	
Resting association	1	2	3	4	5	6	7	9	11	Mean	Pearson´s r^b	P-value^b
M488-M487	~	1,00	0,43	0,00	x	0,67	0,40	~	1,00	0,58	-0,127	0,786
M488-M485	~	0,00	0,29	0,00	x	0,67	0,40	~	1,00	0,39	0,519	0,233
M488-M489	~	0,40	0,43	0,00	x	1,00	0,40	~	0,00	0,37	-0,487	0,268
M488-M484	~	0,40	0,57	0,50	x	0,33	0,60	~	0,00	0,40	-0,523	0,229
M487-M485	~	0,00	0,29	0,00	x	0,67	0,80	~	1,00	0,46	0,556	0,195
M487-M489	~	0,40	0,29	0,00	x	0,67	0,60	~	0,00	0,33	-0,611	0,145
M487-M484	~	0,40	0,00	0,00	x	0,33	0,20	~	0,00	0,16	-0,401	0,373
M485-M489	~	0,00	0,71	0,75	x	0,67	0,60	~	0,00	0,46	0,036	0,939
M485-M484	~	0,00	0,00	0,00	x	0,33	0,20	~	0,00	0,09	0,134	0,775
M489-M484	~	0,20	0,00	0,00	x	0,33	0,20	~	0,00	0,12	-0,239	0,606
Mean	~	0,28	0,30	0,13	x	0,57	0,44	~	0,30		0,054	0,908
Group 3												
	Day										Correlation over time	
Resting association	1	2	3	4	5	6	7	9	11	Mean	Pearson´s r^b	P-value^b
M421-M515	0,00	1,00	1,00	0,33	0,25	0,25	0,25	0,71	0,25	0,45	-0,050	0,890
M421-M518	0,00	0,00	0,80	0,67	0,50	0,25	0,75	0,71	0,75	0,49	0,276	0,440
M421-M273	0,00	1,00	0,40	0,33	0,50	0,25	0,50	0,00	0,00	0,33	-0,401	0,250
M515-M518	0,00	0,00	0,80	0,67	0,00	0,00	0,50	0,57	0,25	0,31	0,071	0,845
M515-M273	0,00	1,00	0,40	1,00	0,25	0,25	0,75	0,00	0,25	0,43	-0,343	0,332
M518-M273	0,00	0,00	0,20	0,67	0,25	0,75	0,50	0,00	0,00	0,26	-0,052	0,887
Mean	0,00	0,50	0,60	0,61	0,29	0,29	0,54	0,33	0,25		-0,122	0,737

~ No joint resting bouts were observed
X No behavioural observations were made
[b]One-tailed

Pack 2 clusters into two distinct subgroups, cluster 1 (M488–M487) and cluster 2 (M485–M489). The resting association was the strongest between M488 and M487 which were housed in different subgroups at the onset of the study. Wild dogs within cluster 2 (M485–M489), on the other hand, were housed in the same subgroups at the onset of the study. M484 rested alone more frequently and avoided the other resting dogs, especially when M485 was present which chased often after M484 during feeding. Pack 3 clustered into two distinct subgroups too, cluster 1 (M421–M518) and cluster 2 (M515–M273). The resting association was the strongest between M421 and M518 which were housed in the same subgroups at the onset of the study. The strength of the resting association between M515 and M273 was comparable with the strength of resting associations between M515 and the other pack members. The strength of resting associations between M273 and the other pack members, on the other hand, was weaker in comparison with the strength of the resting association between M273 and M515. M273 and M515 are therefore paired into a separate cluster.

Table 7 shows the relative strength of individual and mean resting association indices between members of pack 1, 2 and 3 during the first 7 days, and the 9^{th} and 11^{th} day after introduction after regrouping. Pearson's rho indicates the level of increase or decrease in relative strength of individual and mean resting association overtime during the two weeks after regrouping. The relative strength of individual and mean resting associations did not change significantly over time.

4. Discussion

The aim of the current project was to determine the effect of DAP® on stress levels of captive African wild dogs and to evaluate the effect of DAP® on intra-pack aggression and social integration during regrouping in captivity. In experiment 1 the perception of DAP® spray by African wild dogs was evaluated. Although no significant differences in behavioural responses were observed between areas treated with DAP® or placebo spray (possibly due to the small sample size), results are promising and show that in general wild dogs have more interest for areas treated with DAP®. In experiment 2a a non-invasive method for assessing activity stress levels in African wild dogs was validated. The experiment showed that an cortisol-3-CMO EIA can be used to reliably monitor changes in adrenocortical via measures of GCM concentrations in the faeces African wild dogs. Specifically, we found that: (1) Changes in adrenal functioning after adrenal stimulation were reflected by changes in faecal GCM concentrations (2) Application of DAP® collars caused pre-treatment faecal GCM concentrations to decrease substantially. In experiment 3 the effect of the application of DAP® on the behavioural process of regrouping was examined. Relative low frequencies of severe and ritualized aggression were observed within the three wild dog packs with collars during first two weeks after regrouping. DAP® collars did not affect former existing relationships and the strength of resting relationships between pack members did not change over time during the two weeks after regrouping.

Perception of DAP® spray

Maternal odours associated with ventral skin secretions of lactating sows are known to have an attractive effect on piglets (*Sus scrofa domesticus*) (Morrow-Tesch and McGlone, 1990). Pageat and Gaultier (2003) isolated this ventral skin secretion which is now known as maternal pig appeasing pheromone (PAP). PAP has the same biological function in domestic pigs as DAP has in domestic dogs. In several cases it has been noticed that domestic dogs are attracted to electric heated diffusers which administer synthetic DAP® into the air (pers. comn. M.B.H. Schilder). However the attractive effect of DAP® on canid species has never been studied in detail.

In the present study, behavioural responses towards areas treated with DAP® or placebo spray were evaluated to examine if African wild dogs perceive DAP®. The hypothesis was that African wild dogs show more interest for and rest longer near areas treated with DAP®. Differences in occurrence frequencies of behavioural responses were observed between areas treated with DAP® or placebo during the first five minutes after release and during a 1 hour observation period. Interestingly, behavioural responses of the four different wild dog packs were quiet variable. To determine if the wild dogs packs in general responded more to areas treated with DAP® or placebo spray the different examined categories of behaviour were clustered into a single category “behaviour directed towards the treated area. Some pack showed more interest for areas treated with one of both sprays, some packs showed equal interest for both areas and other packs showed no interest at all. Interestingly, in general higher occurrence frequencies of behavioural responses were observed towards areas treated with DAP®. In addition, wild dogs rested longer near areas treated with DAP® spray during a 1 hour observation period in two of the four tested packs.

In summary, this is the first study that investigated the potential attractive property of appeasing pheromones in a canid species. Although no significant differences in behavioural responses or duration of resting behaviour were observed between areas treated with DAP® or placebo spray, in general wild packs showed more interest for areas treated with DAP®. To further confirm these promising results that suggest DAP® perception in African wild dogs additional trials are needed. Importantly there was a large inter-subject variability in the behavioural responses towards the treated areas and this may be explained by the volatile character of the pheromone. The sprays may evaporate too rapidly in the atmosphere before clear differences in behavioural response towards both sprays can be observed. It is important to take this into account in future studies and use of electric heated diffusers which administer synthetic DAP® or placebo spray continuous into the air, may ensure a more constant exposure to DAP® and Placebo spray and may improve the current experimental design. Alternatively African wild dogs may not perceive DAP® consciously and behavioural responses /attractive effects cannot be ascribe to the perception of the pheromone but are caused by the perception of the solvent (Pers. comm. P. Pageat).

Physiological validation of a cortisol-3-CMO EIA

Cortisol and corticosterone specific radioimmunoassays (RIAs) for monitoring plasma cortisol concentrations and faecal corticoid metabolites levels in African wild dogs have been validated in previous studies (de Villiers et al., 1995; Monfort et al., 1998). Although RIA is reliable and accurate, this technique utilises radioisotopes. Exposure to radioactivity is a risk which restricts its application to specialized laboratories. Enzyme-linked immunosorbent assay (ELISA), on the other hand, has many of the advantages of RIA (e.g., sensitivity, ease of handling multiple samples) without the disadvantages of dealing with radioactivity. In addition, the expenses of performing EIA are lower and procedure times are reduced (Bellem et al., 1995; Sarkar et al., 2007). To our knowledge so far no EIA has been validated for non-invasive monitoring of stress responses in African wild dogs via faecal sampling.

In the present study an adrenal stimulation test (ACTH Challenge test) was performed to validate an cortisol-3-CMO EIA for monitoring GCM levels in faeces of African wild dogs. As a result of Adrenal stimulation we were able to detect a rise and fall in faecal GCM concentrations using the cortisol-3-CMO EIA. Although the identity of the detected faecal GCMs remains unknown, it physiological relevance as indicator of adrenocortical activity was demonstrated. Results of Monfort et al. (1998) indicate that neither cortisol nor corticosterone are the main GCMs in the faeces of African wild dogs, a single peak of immunoreactivity eluted between cortisol and corticosterone references tracers (Monfort et al., 1998).

Administration of exogenous ACTH induced an 190–268% increase of faecal GCM levels compared to baseline levels. Compared to previous studies that performed an adrenal stimulation test on canid species this study found relative low peak GMC levels in the faeces. Schatz and Palme (2001), who used the same dose (25 IU) of synthetic ACTH and the same cortisol-3-CMO EIA, found higher peak GMC levels (range 129-3482%) in faeces of domestic dogs. Studies on other canids, including African wild dog (range 1000–3000%, 400 IU ACTH, (Monfort et al., 1998)), red wolf (*Canis rufus*) (range 405-1160%, 140 IU ACTH; (Young et al., 2004)) spotted hyena (*Crocuta crocuta*) (range 1300–4500%, 200 IU ACTH, (Goymann et al., 1999)), and brown hyena (*Hyaena brunnea*) (range 388-2682%, 50 IU ACTH, (Hulsman et al., 2011)), found on average higher peak GMC levels too. However, these used different types of cortisol, corticosterone, and 11-oxoetiocholanolone EIAs and/or RIAs and a higher dose of synthetic ACTH for adrenal stimulation. Compared to these studies, this study used a relative low dose (25 IU) of synthetic ACTH. This may explain the rather low relative peak concentrations observed in this study.

Peak levels of faecal GCMs were observed 3-8,75 hours after ACTH injection. In the present study, the time delay between ACTH administration and the appearance of peak GCM levels in faeces was much smaller then the time delay (24-30 hrs after ACTH administration) detected in previous study on African wild dogs (Monfort et al., 1998). The time delay observed by Monfort et al. (1998) falls within the range of time delay previously reported for other canid species, including domestic dog (8-71 hr, (Schatz and Palme, 2001), gray wolf (*Canis lupus*) (16–20 hr, (Sands and Creel, 2004)), red wolf (72 hr; (Young et al., 2004)), spotted hyena (16–50 hr, (Goymann et al., 1999)) and brown hyena (25-40 hr; (Hulsman et al., 2011)). The variation between the time delay observed in this study and the timely delay observed by Monfort et al., (1998) is likely to be result of the diet of the African wild dogs in this study. The time delay between ACTH administration and the appearance of peak GCM levels in faeces reflects the gastrointestinal transit time ((Palme et al., 2005; Touma and Palme, 2005). The diet probably causes osmotic diarrhoea resulting in a faster gastrointestinal transit time and thus in a smaller time delay.

In summary, an cortisol-3-CMO EIA was validated for monitoring GCM levels in faeces of African wild dogs and the physiological relevance of faecal GCM levels as indicator of adrenocortical activity for African wild dogs was demonstrated

The effect of DAP® on baseline stress levels

To date few studies have been published regarding the effect of synthetic appeasing pheromones on acute physiological stress responses and baseline stress levels. Yonezawa et al. (2009) investigated the effects of synthetic Pig appeasing pheromone (PAP ®) on baseline stress levels and social stress responses in miniature pigs. After a two-week exposure to synthetic PAP® no differences in baseline saliva cortisol levels of miniature pigs were found between the PAP®-treated group and placebo-treated group. However, the expected social stress response after audiovisual confrontation and physical confrontation was lower in the PAP-treated group in comparison with the placebo-treated group. Siracusa et al., (2010) evaluated the effect of DAP® exposure on the perioperative stress response in domestic dogs undergoing elective orchiectomy or ovariohysterectomy. Exposure to DAP® spray 30 minutes before and after surgery did not significantly affect the generally observed differences between preoperative and postoperative saliva cortisol concentrations in domestic dogs.

In the present study, exposure to synthetic DAP® did affect pre-treatment stress levels of three female African wild dogs. This confirms the hypothesis that application of DAP® collars reduces baseline stress levels of the single housed African wild dogs. In two of the three subjects a substantial reduction of mean faecal GCM concentrations was observed after application of DAP collars. During the DAP®-treatment period mean faecal GCM concentrations reduced to 68% and 82% compared to corresponding mean pre-treatment levels. In one dog no substantial difference was found. However, during the pre-treatment period a relative low mean faecal GCM level was found for this wild dog compared to the baseline period. This may explain the absence of a difference between the observed mean faecal GCM level of the pre-treatment and DAP-treatment period. The faecal GCM levels of the three wild dogs decreased to some extent over time during the DAP®-treatment while a slight increase was observed during the pre-treatment period.

In summary, substantial differences were found between mean faecal GCM of the pre-treatment and DAP®-treatment period. In addition, faecal GCM levels decreased to some extent over time, although not significantly (possibly due to the limited number of samples), during the DAP®-treatment period. The findings of this study are in contrast with the results of (Yonezawa et al., 2009) who found no differences in baseline saliva cortisol levels of miniature pigs after exposure to synthetic appeasing pheromone. An important difference however is that this study used a single-subject study design and (Yonezawa et al., 2009) used a placebo-controlled study design.

Future studies are necessary, testing larger sample sizes, to extend these promising preliminary results. These could be improved by extending the pre-treatment period and DAP®-treatment period to control for a potential effect of immobilisation ((de Villiers et al., 1995)) or to ensure complete recovery of the HPA axis responsiveness after DAP® collar application for which immobilisation was necessary. Alternatively, wild dogs could be exposed to DAP® using a plug in evaporator when indoor (night) enclosures are present.

The effect of DAP® on the behavioural process of regrouping

The present study evaluated the effect of the application of DAP® on the behavioural process of regrouping. The behavioural process of pack formation of the three regrouped wild dogs pack was characterized by relative low frequencies of severe and ritualized aggression. These findings suggest a relatively low level of agonistic arousal and tension between pack members during the process of pack formation. In contrast, relatively high frequencies of dominant behaviour were observed during first days of pack formation. However, dominant behaviour declined to relative low levels which suggest that reciprocal hierarchal relationships were established soon after regrouping and were maintained occasionally via dominance (threat) and submissive displays. Relative high levels of affiliative behaviour were observed which decreased to some extent over time. The presence of relative high levels of affiliative behaviour in the three packs throughout the entire observation period suggests that reciprocal relationships between pack members were maintained through amiable interactions too.

Throughout the entire observation period relative high levels of ritualized aggression were observed in pack 3 compared to levels observed in pack 1 and pack 2. The relative young age of (some) members of pack 3 compared to members of pack 1 and 3 may explain the relative high levels of ritualized aggression observed within this pack. de Villiers et al. (1997) found that younger wild dog males often employ a relative active dominance style, characterised by relative high levels of (ritualized) aggressive (threat) behaviour, compared to older males which use a passive dominance, characterised by low levels of (ritualized) aggression (threats) and high levels of affiliative behaviour, to elicit submission from a subordinate. Younger wild dog males tend to use proportionately a more active dominance style during agonistic interactions. These age-related behavioural differences in dominance style may reflect social skilfulness and confidence regarding social status which improves with age. Another explanation for the observation of relative high levels of ritualized aggression is the sub-optimal pack composition of this artificial created pack. The pack comprised two unrelated wild dogs and two genealogical related wild dogs from different litters.

Furthermore relative high levels of social play were observed within pack 3. Social play is characteristic for young animals too and it has been suggested that through social play young animals learn their role in society. Social play provides information about the rank and strength of other group members and enables young animals to develop their social communication system and social skills (Poole, 1985). In canids, play appears to be important in learning to inhibit or rather control the intensity of the bite and facilitates the formation and continued maintenance of social relationships (Bekoff, 1974; Fox, 1971). Bite intensity provides a congener information about the intensity of agonistic arousal and increases if the adversary does not remain passive but retaliates (Fox, 1971).The relative young age of (some) members of pack 3 may therefore also explain the relative high levels of social play

During the feeding period relative high frequencies of ritualized aggression and dominant behaviour were observed. The relative high frequencies of ritualized aggression dominant behaviour during the feeding maybe caused by increased food competition within the packs. In pack 2 for example one subordinate wild dog chased often after the most submissive wild dog and was mainly responsible for high levels of ritualized aggression observed during the feeding period. Several studies on primates showed that the distribution of food in provisioned groups can influence levels of aggressive behaviour (Boccia et al., 1995; de Waal, 1984; Gore, 1993; Southwick et al., 1976). In these studies higher levels of aggressive behaviour were observed when food was provided in a clumped manner compared to trials in which food provided in a dispersed manner. In this study the diet African wild dogs consisted mainly of pellet shaped domestic dog food provided by clumped manner in which food (in one or two mangers). Food provide in a clumped manner can be monopolised relative easily by dominant animals through active defence and animals with a relative low social status have therefore less access to food resources. Experience of food shortage by subordinates may enhance food competition between subordinates. The clumped manner in which food was provide may therefore explain the relative high levels of ritualized aggression and dominant behaviour observed during feeding. Sufficient distance between food resources (opposite corners of the enclosure) and / or multiple food resources in large enclosure diminishes the possibility to monopolize food resources. Furthermore, food provided in a dispersed manner or food which can be moved by the wild dogs (e.g bones, pieces of meat) may lower observed levels of ritualized aggression and dominant behaviour.

The strength of resting associations was analysed and members of the three regrouped packs clustered into one or more subgroups. The clustering roughly corresponded with the genealogical relationship between pack members (Pack1) or corresponded with previous relationships which already existed before the wild dogs were separated because of severe aggression within the pack (Pack 1 and Pack 2) (Pers. Comn. Ann van Dyk Cheetah Centre - De Wildt). The strength of resting association between members of the three regrouped wild dog packs did not change significantly overtime. This suggest that exposure to DAP® collars did not affect relationships between pack members. Resting associations between separated pack members which were housed in the same subgroup at the study onset were not stronger. This suggests that temporally separation of the pack members, on the other hand did not affect the existing relationships separated pack members either.

In summary, the behavioural process of pack formation of the three regrouped wild dogs pack was characterized by relative low frequencies of severe and ritualized aggression. These findings suggest a relatively low level of agonistic arousal and tension between pack members during the process of pack formation. DAP® collars did not affect existing relationships and the strength of resting relationships between pack members over time. To determine whether the relative low frequencies of severe and ritualized aggression observed can be ascribed to the application of DAP®, it would be interesting to extend the study and to perform a similar detailed quantitative analysis of the behavioural process of artificial pack formation during a pack formation event in which DAP® is not applied.

Although there are some studies regarding artificial pack formation and reintroduction of captive African wild dogs it is difficult to compare the results of these studies with the result of this study. The present study gives a detailed quantitative description of the behavioural process of pack formation during the first two weeks. In contrast, previous studies provide primarily qualitative descriptions of the behavioural process of pack formation over several months and the long term outcome (Graf et al., 2006; Gusset et al., 2006). Even though considerable aggression and disruption to the social structure is observed in these studies no or only brief quantitative descriptions of behavioural interactions are reported.

Another problem this study encountered was that many studies do not provide clear definitions of the terms ‘aggression’ and ‘dominance’ and restrict themselves to basic terminology (e.g. aggression, dominance behaviour, submission, affiliative behaviour). In addition, some studies make no clear distinction between aggression and dominance and use these terms interchangeably. Some authors for example restrict the use of the ‘term’ aggression to describe physical attack while others include aspects of more mild threatening behaviour (threat displays) associated with lower levels of agonistic arousal. Although some studies, zoos and breeding centres report considerable levels of intra-pack aggression, it is currently difficult to interpret and compare results because often no detailed description and occurrence frequencies of the observed behaviour are reported. Often it is not clear whether mainly severe aggressive behaviour, ritualized / stylised aggressive behaviour or dominant behaviour is observed within the packs. Therefore, it is difficult to determine and compare the effectiveness of methods to prevent or reduce stress and intra-pack aggression during artificial pack formation. Because there is little consistency and agreement in the literature about terminology concerning agonistic interactions it is important that authors provide a clear concise descriptions for the terminology they use which consider the species of interest and/or the experimental context.

Conclusion

In conclusion the findings of this study indicate that: 1) ehavioural responses towards areas treated DAP or placebo sprays while tested in an outside area are indicative, although insignificantly in the sample size of this study, for the perception of DAP® by African wild dogs. Importantly wild dog packs in general showed more interest for areas treated with DAP® which is promising, 2) the cortisol-3-CMO EIA is a suitable EIA for monitoring GCM levels in faeces of African wild dogs, 3) faecal GCM levels are physiologically relevant indicators of adrenocortical activity for African wild dogs, 4) DAP® collars substantially reduce pre-treatment stress levels of female African wild dogs 5) relative low frequencies of severe and ritualized aggression are present during regrouping events when DAP® spray and DAP® collars are applied before regrouping, and 6) the strength of (existing) resting relationships between pack members is not affected by DAP®

Future studies

This study provides new insights in the field of African wild dog conservation research, suggesting that the application of DAP® spray and DAP® collars prior to social introduction of African Wild Dogs contributes to the reduction of social stress and intra-pack aggression during this event. Based on these promising findings it would be highly relevant to extend the sample size and evaluate more social introductions of African wild dogs in which DAP® spray and/or DAP® collars are applied. However, to confirm, elaborate and implement these initial results, support and cooperation of more zoos and breeding centres is indispensable. Because the current study only included re-groupings of African wild dogs of the same sex additional trials should be conducted to determine whether the application of DAP® is equally effective during the social introduction of African wild dogs of opposite sex. Furthermore, future studies should take into account that behavioural data regarding the behavioural process of social introduction is collected and evaluated in a consistent and uniform manner. In order to do so, it is important that future social introductions of African wild dogs, in which DAP® is applied, are evaluated by a single behavioural biologist, who has in-depth knowledge of the species social behaviour.

Besides the application of DAP®, researchers should also evaluate the introduction methods for social African wild dogs to determine whether these can be improved as well. Currently management decisions regarding translocations and social introductions of African wild dogs Zoos and breeding centres are based primarily on subjective intuitive assessments of animal curators and experienced animal husbandry staff. Although social introductions of African wild dogs without the use of rigorous protocol have been successful, in general wild dog introductions are cumbersome and the risk / potential for significant social stress and excessive intra-pack aggression is high. A more systematic planned stepwise introduction method, in which empirical data (e.g. behavioural criteria) is used for decision-making regarding the introduction process, could be a useful method to reduce social stress and/or aggressive interactions during and after social introduction of African wild dogs (Burks et al., 2001; Burks et al., 2004; Powell, 2010; Winslow et al., 1992). A social acclimatization period in adjacent enclosures before full physical social introduction for example, in which visual, audial and olfactory contact can be established, may considerably reduce potential stress and aggression during the introduction event. African wild dogs show different types of agonistic (aggressive) behavioural which are associated with different levels of agnostic arousal (aggression). Without thorough understanding of the social behaviour of African wild dogs it is hard to distinguish normal dominant behaviour and ritualized aggression from excessive (severe) aggression and make objective assessments about the behavioural process during the social introduction and the final outcome. Close collaboration between behavioural biologists, animal curators and husbandry staff could lead to the development of such a systematic stepwise introduction protocol for the African wild dog.

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7. Appendix

Supplementary table 1. Ethogram of behavioural responses performed within 1 ADL of the treated area	
Behavioural response	**Code**
Sniffing at area	SA
Resting	RE
Urinating	UR
Licking mandibular area	LM
Panting with tongue out of the mouth	PA
Standing*	STA
Flehmen response*	FR
Licking at area*	LA
Defecating*	DE
Scent marking (rolling)*	SM
Area defence*	AD
*Behavioural response which did not occur during the experimental trial	

Supplementary table 2. Sample list with corresponding sample collection times (Part 1)		
Studbook ID	**Container ID (Animal and sample number)**	**Sample collection times (h) before / after ACTH administration**
F423	B 22	-20,25
	B 25	-17,25
	B 28	-15,5
	B 30	3,5
	B 33	8,25
	B 36	11,25
	B 40	32
	B 43	35
	B 45	47,75
	B 48	54,75
	B 51	56,5
	B 52	58,75
	B 53	72,75
	B 56	80,25
	B 59	104,25
	B 62	168,25
	B 65	175,5
	B 68	179,25
	B 70	191,75
	B 73	198,75
	B 76	201,75
	B 79	216
	B 82	224,25
	B 85	226,75
	B 87	240,5

Supplementary table 2. Sample list with corresponding sample collection times (Part 2)		
Studbook ID	**Container ID (Animal and sample number)**	**Sample collection times (h) before / after ACTH administration**
F424	E 23	-20
	E 26	-16
	E 31	3,25
	E 34	8,5
	E 38	23,75
	E 41	33
	E 46	47,75
	E 49	56,5
	E 54	72,75
	E 57	81,75
	E 60	105,75
	E 63	168,25
	E 66	175,5
	E 69	179
	E 71	191,75
	E 74	199,25
	E 77	202,75
	E 80	216
	E 83	224,25
	E 86	227,25
	E 88	240,5
F322	F 24	-19,75
	F 27	-15,25
	F 29	-13,75
	F 32	3
	F 35	8,75
	F 37	11,25
	F 39	23,75
	F 42	30,75
	F 44	33,25
	F 47	47,75
	F 50	57,25
	F 55	72,75
	F 58	82
	F 61	106
	F 64	168,25
	F 67	176,5
	F 72	191,75
	F 75	199,5
	F 78	202,25
	F 81	216
	F 84	224,5
	F 89	240,5

Supplementary table 3. Ethogram of social interactions of the African wild dog (Part 1)

Behavioural category	Behaviour	Code	Description
Postural performance (Indicative for the rank of the actor with respect to the recipient during a social interactions)	High posture	HP	High head position, erected tail, ears kept up and the legs and back straight (Dominant).
	Neutral posture	NP	A relaxed and intermediate body posture (Assertive).
	Low posture	LP	Head low, tail bent downwards or between the legs ears folded backwards (Submissive).
Severe aggressive behaviour	Assault	AS	A direct, rapid full speed approach at another dog often followed by aggressive physical contact.
	HP / LP Severe bite	SBI	Close jaws and teeth on another getting a strong hold of any part of the opponents body e.g. legs, tail, throat or head. Biting with full strength (uninhibited).
Ritualised aggressive behaviour	Chase away	CW	Walking/running in pursuit to elicit a flee response from the recipient.
	Push down	PB	Pressing down the recipient by an inhibited bite in the neck / Getting another dog down to the ground.
	Embrace	EM	Embracing the neck of another dog from the front, the recipient can stand on for or two hind legs.
	Muzzle bite	MBI	The actor seizes of the recipient between its jaws from the side or from above and holds it gently for a while (inhibited).
	HP / LP Scruff bite	SCBI	Scruff orientated inhibited bite.
	HP/ LP Snap	SNP	Close jaws and teeth on another without physical contact.
Dominant behaviour	HP/ / LP Threat (bared teeth)	TH	All degrees of facial display in which the mouth is more or less opened, the teeth are bared and forehead and the nose are wrinkled. Growling is often heard and locomotion is typically absent. The body posture and position of the mouth and ears may vary.
	Aggressive vocalization	AV	A growling vocalisation.
	HP / LP Scruff orientated approach	SOA	Scruff orientated approach which does result not resulting in scruff orientated biting.
	Stalk approach	SA	Actor stands or slowly approaches the recipient with a prowling posture; that is with the head and neck in a straight line below the shoulder, the ears folded back, the tail relaxed or in a straight horizontal line below the shoulder and the ears folded back.
	Food approach	FA	Locomotion oriented towards the recipient diminishing the distance while looking at him in the context of food acquisition (take over).
	Intervention by approach	IVAP	The actor stops an interaction between two other interactors by approach / stand in between in high position.
	HP Head turning	HT	Head turning and avoidance of eye contact avert gaze which does expose the neck region to the recipient
	HP stand	ST	Stand in a high position.
	Fixate	FI	Looking straight at a recipient from a distance, motionless, in high posture and ears forward (intense fixed gaze).
	Mark over urine mark or food	MO	Actor secretes a small amount of urine over a previous urine mark or food.
	Freezing	FR	The actor stands stiff with the head straight to ground and the eyes fixated, either on the ground or on the recipient; the behaviour is mostly shown as reaction on food approach.
	Inguinal-genital inspection	IGI	Actor initiates inguinal contact and investigates recipients genitals. Recipient remains passive. The actor is dominant over recipient.
	Point	PO	The actor directs with an abrupt movement of his head or a short jump, towards the recipient.

Supplementary table 3. Ethogram of social interactions of the African wild dog (Part 2)

Behavioural category	Behaviour	Code	Description
Play fighting	Play Solicit	PS	The actor initiates play fighting by soliciting behaviour e.g. nose pushing or tugging the recipients fur with an inhibited bite.
	Fur bite	FB	The actor tugs the recipients fur by a inhibited bite.
	Play fighting	SP	Playful non-competitive fighting in which attacker and defender exchanges roles and no winner or loser emerges. Interactions rarely include behaviours which can inflict injury.
	Play Chase	PC	The actor follows in a fast pursuit the recipient, who tries to escape by abruptly changing the direction. The role of follow and escape may change during the course of action.
	Play Wrestle	PW	This behaviour involves all play situations, in which the actor shows inhibited bite movements towards the recipient, while the actor keeps constant eye contact with the recipient.
Submissive behaviour	Escape / flight	ES	To run away from another dog, often shown during conflicts .
	LP Retreat	RE	Take more distance for another dog after being approached.
	Shrink back	SB	The actor jumps back from the recipient, after being approached by him.
	Avoid	AVO	Stand a side for another dog after being approached.
	Active submission	ASM	A behavioural complex in which the actor actively seeks contact with a recipient by approaching it in a crouched manner with curved back and bent legs, while the tail is bent down, often wagging, and while the ears are folded back. From this position the
	Passive submission (Go down)	PSM	The actor lies on its side or half on its back, exposing chest and belly towards the recipient. The tail is drawn in between the hind legs and the ears are flattened backwards. The actor remains completely motionless. In African wild dogs (Go down)
	LP Head turning	HT	Head turning and avoidance of eye contact avert gaze which does expose the neck region.
	Flattened Ears	EB	Ears pulled back, flattened.
	Tail between the legs	TBL	Tail between the legs.
	LP standing	ST	Stand in a low position.
	LP approach	AP	Locomotion towards the recipient, diminishing the distance while looking at him.
	LP snout	SN	The actor brings his nose close or pushes it towards the nostrils of the recipient.
	Submissive vocalisation	SV	Twittering, whimpering, yelping, whining vocalisations.
	Present body	P	Actor rolls on his side in front of the recipient or roll towards him awaiting inspection
	Food solicit	SO	The actor approaches or walks in parallel with the recipient while begging for food and trying to reach for his mouth corners there is some resemblance with "greeting", but the context is different and the behaviour is not likely to be reciprocated
	Hoo call	HC	Heard when dogs become separated while hunting and indicative for distress.

Supplementary table 3. Ethogram of social interactions of the African wild dog (Part 3)

Behavioural category	Behaviour	Code	Description
Affiliative behaviour	Close contact	CC	Sit, stand or lie in close to the recipient.
	NP approach	AP	Locomotion oriented towards the recipient diminishing the distance while looking at him.
	NP snout	SN	The actor brings his nose close or pushes it towards the nostrils of the recipient.
	NP face lick	FL	The actor licks the nose and mandibular region of the recipients snout.
	Pass under head	PUH	The actor passes from a lateral side close under the head of the recipient, usually in a somewhat crouching manner; often a short nose chin contact with recipient is evident.
	Head under	HU	Actor pushes with his head towards the ventro-lateral side of the recipient, occasionally lifting with his head; the recipients back quarters from the ground.
	Fur sniff / licking	FSL	Sniffing / licking the recipients fur.
	Paw /head on	PHO	All body contacts longer than momentary duration in which the actor places a paw or head on the back of the recipient.
	Grin	GR	The mouth corners remain retracted and the mouth may be slightly open so that the teeth's become visible. The behaviour occurs mostly in combination with giggle, a high tone level staccato rhythm.
	Giggle	GI	Vocalisation characterised by a high tone level staccato rhythm.
	Greeting	GC	Actor stands or walks in parallel with the recipient, tries to contact this muzzle, and performs a complex of behaviours including food solicit, inspection, giggle and grin.
	Parallel walk or run	PW	Walk or run near by another dog sometimes with physical contact within a distance of one body length.
	Reagguration	RE	Regurgitation is the expulsion of undigested food from the mouth, pharynx, esophagus.

Supplementary table 3. Ethogram of social interactions of the African wild dog (Extra)

Behavioural category	Behaviour	Code	Description
Social investigation	Present body	P	Actor rolls on his side in front of the recipient or rolls towards the recipient awaiting inspection.
	Fur sniff / licking	FSL	Sniffing / licking the recipients fur.
	Inguinal-genital inspection	IGI	Actor initiates inguinal contact and investigates recipients genitals. Recipient remains passive. Actor dominant is over recipient.
	Head under	HU	Actor pushes with his head towards the ventro-lateral side of the recipient, occasionally lifting with his head; the recipients back quarters from the ground.
	Tail position	TP	The actor stands behind the recipient with his head directed towards recipients ano-genital region.
	Tail sniff	TS	Self-explanatory.
Sexual behaviour	Ride-up/ Mounting	RU	Resting forelegs or paws on or across the back of another dog (sometimes bite in the neck or thrusting pelvis but no intromission).
	Thrust	TH	Oscillating back and forth movements of the pelvis which one dog makes while mounted on another.
	Tie	TI	The locking together of male and female canines at the genitals during copulation.
	Intromission /Penetration	IP	Penetration of the vagina by the male's penis.
	Copulation	CO	The act of mating, resulting in a copulatory tie for at least one minute.