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Research

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Efficacy of Natural Herbal Formulation against Acute Hepatopancreatic Necrosis Disease (AHPND) causing *Vibrio parahaemolyticus in Penaeus vannamei*

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ABSTRACT

A formulation was developed using combination of blended natural essential oils as an anti-*Vibrio parahemolyticus* causing acute hepatopancreatic necrosis disease (AHPND) candidate. *Lavandula latifolia, Pinus sylvestris, Jasminum officinale*, *Citrus limon*, *Prunus avium, Viola odorata, Gardenia jasminoides, Cocos nucifera, Rosa damascene* and *Eucalyptus globulus,* mixed together to develop as anti-*V. parahemolyticus* product. The treatment group was fed on essential oil mixed feed whereas control group were fed on the regular feed throughout the experiment. The shrimp of both treatment and control were challenged by immersion method at day 8. The cumulative AHPND-gross sign appearance in positive control reached up to 95% at dpi 10 whereas no gross sign appeared in treatment and in negative control. The cumulative mortality reached up to 46.7% at dpi 10 in positive controls whereas no mortality recorded in treatment and in negative control. The *V. parahaemolyticus* isolated from the hepatopancreas of infected shrimp matched 100% with the existing AHPND strain. The trial results show that the developed natural herbal formulation has significant effect against AHPND in a controlled condition.

KEYWORDS: Acute hepatopancreatic necrosis disease (AHPND); *Vibrio parahemolyticus*; Essential oil blend; anti-AHPND feed.

ABBREVIATIONS: AHPND: Acute Hepatopancreatic Necrosis Disease; SPF: Specific Pathogen Free; DO: Dissolved Oxygen; WSSV: White Spot Syndrome Virus; IMNV: Infectious Myonecrosis Virus; IHHNV: Infectious Hypodermal and Haematopoietic Necrosis Virus; TSV: Taura Syndrome Virus; YHV: Yellow Head Virus; PCR: Polymerase Chain Reaction; BLAST: Basic Local Alignment Search Tool.

INTRODUCTION

The acute hepatopancreatic necrosis disease (AHPND) has affected shrimp farming in several countries, like, Vietnam, Malaysia, Thailand, Mexico and in Philipines.[1-6] The unique symptoms and characteristics of this disease consisting of massive sloughing of hepatopancreas epithelial cells.[4] The external symptoms in infected shrimp like, empty stomach, bluish body color and shrunken hepatopancreas could be observed. The rate of mortality is significantly higher on the 1st 3 days of infection. The AHPND appear in the culture ponds from 8-45 days of stocking.

The *Vibrio parahaemolyticus* was identified as causative agent of AHPND by Tran et al.[7] It carries a plasmid (pAP1) of approximately 69 kbp. This plasmid contains 2 genes which

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produce toxins. These 2 genes act together to cause AHPND in shrimp. The susceptible known species are *Penaeus vannamei*, *P. monodon* and *P. chinensis*.[8]

There are several efforts done to minimize the effect of pathogen using herbal products. Other authors[9] showed the anti-microbial effects of 2 essential oils (EOs) Vibrio concentrations in the rearing water of *Penaeus monodon*.

The proposed trial was designed with the objective to determine the efficacy of essential oil enriched shrimp feed against *Vibrio parahemolyticus* in *Penaeus vannamei*.

MATERIALS AND METHODS

Bioassay Lab and Glass Aquaria

A bioassay trial was set-up using 6 aquaria each for experimental groups as well as for control groups. The specific pathogen free (SPF) juvenile shrimp 10 each in number were maintained in 6 aquaria each. Each aquarium was filled with 5 litre of seawater provided with required dissolved oxygen (DO) supply.

Location and Time Period

The experiment was conducted in bio-secured laboratories at Ben Tre Aquaculture Station, Binh Dai, and Extension Department, Vietnam in the period of September 16, 2014 to October 30, 2014.

Experimental Shrimp

Shrimp of mean body weight 0.23 to 0.33 g were used to conduct the trial. The available size of SPF Shrimp (PL 10) were procured from a bio-secured Hatchery (Ca Dec Seed Production Centre, Ben Tre, Vietnam) and reared in 500-litre capacity tank for 10-15 days.

Shrimp Screening and Acclimatization

The shrimp were screened at Government disease diagnostic center (RAHO), Ho Chi Minh City and CÔNG TY CO PHAN DICH VU THUY SAN THANH LOAN Disease Diagnosis Lab at Ho Chi Minh City for pathogen of shrimp i.e. white spot syndrome virus (WSSV), Infectious myonecrosis virus (IMNV), infectious hypodermal and haematopoietic necrosis virus (IHHNV), taura syndrome virus (TSV), yellow head virus (YHV) and *Vibrio* spp. prior to start of the trial.

Shrimp Food

Shrimp feed were produced at Feedmill, Lampung of PT. Central Proteinaprima Tbk. The feed types were as followed, post-larvae feed (PL 03:250-400 micron) and shrimp feed (CP 001:0.425-0.75 mm and CP 02:0.71-1 mm) as per the requirement of experimental shrimp. The anti-AHPND essential oil formulation were developed by combining the essential oil blend extracted from the following 10 plants, *Lavandula latifolia, Pinus sylvestris, Jasminum officinale*, *Citrus limon*, *Prunus avium, Viola odorata, Gardenia jasminoides, Cocos nucifera, Rosa damascene* and *Eucalyptus globulus.* Using expeller-pressing method (Anderson International Corp, OH, USA) performed the oil extractions from the selected plants.[10] The essential oil blend were mixed with the feed in required amount. The basic formulation of both the feed was same except essential oil mixed in the experimental feed.

AHPND Disease Challenge Procedure

The immersion method of challenge was applied in the trial.[11] The isolated purified bacteria, *Vibrio parahemolyticus*, strain VP A/3 (procured in August 2014 from University of Arizona, USA) were utilized for the trial. The *V. parahemolyticus* was grown up to the density of 10^7 CFU/ml in Tryptose Soy Broth (TSB) and then 5 ml of it was poured in each tank. The final *V. parahemolyticus* concentration in the tank water was 10^5 CFU/ml. The 5 ml blank TSB was applied in the negative control tanks (Table 1).

Post-Challenge Observation

EMS gross sign observation: The challenged shrimp were observed for body color, hepatopancreas color and shape and feed consumption rate and cumulative mortality. The severity level of infection was categorized into:

- Medium level G2: Hepatopancreas color light pale, yellow within hepatopancreas connective tissue capsule, size of hepatopancreas normal and no sign of atrophy;
- Severe level G3: Hepatopancreas color pale, yellow or white within hepatopancreas connective tissue capsule, significant sign of atrophy in hepatopancreas (shrunken).

Group	Replicate	MBW (gr)	Density/5 L	Bacteria Density (CFU/mL)	Challenge methods	Feed	Water Exchange
Positive Control	6	0.23-0.33	5	10^7	Pour 5 mL of *Vibrio parahaemolyticus* in each tank	Regular	20%/ day (no water exchange during challenge)
Treatment						Essential oil enriched	
Negative Control					Pour 5 mL of sterile TSB solution in each tank	Regular	

Table 1: Experimental design of immersion challenge trial.

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Lab confirmation: The AHPND was confirmed by typical gross sign appearance, by polymerase chain reaction (PCR) analysis and sequencing analysis.

Vibrio parahaemolyticus Isolation and Sequencing Analysis

The *Vibrio parahaemolyticus* was isolated from the hepatopancreas of challenged shrimp using Chrom-Vibrio agar. The colonies of *V. parahaemolyticus* spp. appeared mauve color. Further, sequencing of obtained *V. parahaemolyticus* was carried out. The primers utilized were AP3 Reverse and AP3 Forward[12] with a base length of 236 bp. The analysis of sequencing was performed at First Base, Singapore.

Statistical analysis: Statistical analysis were done by analysis of variance (ANOVA) with $p<0.05$ confidence level.

RESULTS

Shrimp Gross Sign Appearance

The positive control shrimp started showing the clear symptoms of AHPND after 18 to 22 hour of challenge. The stomach was empty and significant drop in the lipid droplets in hepatopancreas.

Feeding Rate

The feed consumption dropped significantly after challenge in positive control (about 50%). It showed the stress in shrimp. The shrimp start recovering in the treatment group from dpi 3 onwards (Figure 1).

Cumulative Mortality

The cumulative gross sign appearance in positive control reached up to 56% by day 3 of challenge (Figure 2). The typical gross sign of AHPND, like, shrinkage of hepatopancreas, reduction in lipid content and empty stomach, started appearing from the day of challenge. The cumulative mortality rate was 46.7% at dpi 10 after challenge in positive control whereas no mortality in treatment group and in the negative control group (Figure 3). The moribund and dead shrimp had clear symptoms of AHPND.

Vibrio parahaemolyticus Isolation and Basic Local Alignment Search Tool (BLAST) and Sequence Alignment

The extracted *Vibrio parahaemolyticus* from the stomach of positive control shrimp were sequenced (Table 2) and aligned (Figure 4).

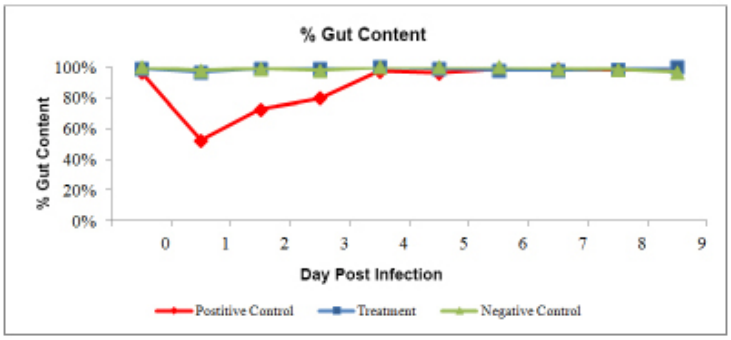

Figure 1: Daily feed consumption rate in percentage (%).

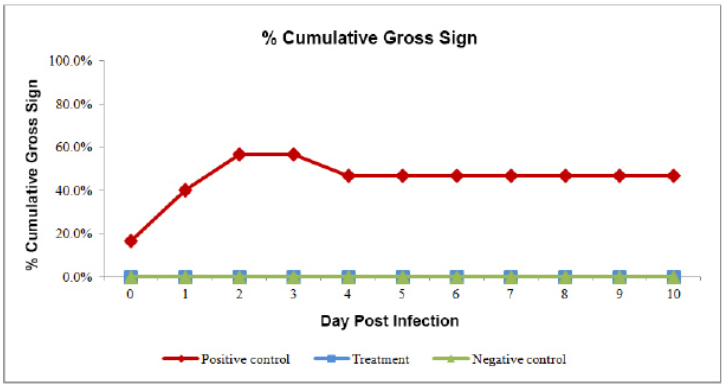

Figure 2: Cumulative gross sign percentage of AHPND in trial groups.

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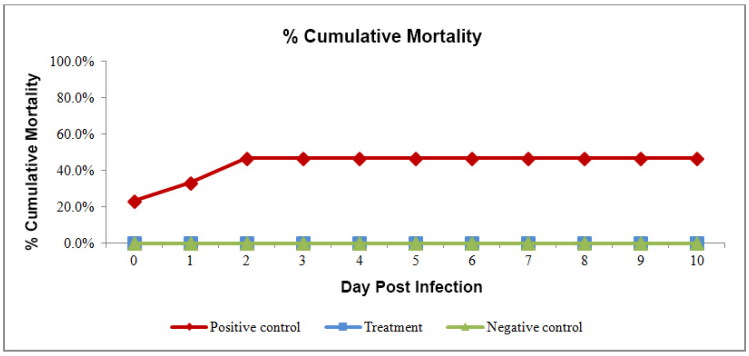

Figure 3: Cumulative mortality percentage of trial groups.

Description	Max Score	Total Score	Query Cover	E value	Ident	Accesion
Vibrio parahaemolyticus strain 3 HP plasmid pVA1, complete sequence	425	425	99%	2e-115	100%	KP324996.1
Vibrio parahaemolyticus genes for hypothetical proteins, JHE-like toxin PirA-like, JHE-like toxin PirB-like, complete cds	425	425	99%	2e-115	100%	AB972427.1
Vibrio parahaemolyticus strain 13-028/A3 plasmid pVPA3-1, complete sequence	425	425	99%	2e-115	100%	KM067908.1
Vibrio parahaemolyticus strain 20130629002S01 putative VP19 protein (vp19) gene, complete cds	425	425	99%	2e-115	100%	KM035408.1
Vibrio parahaemolyticus plasmid pVPA3-1 DNA, putative toxin region	348	348	84%	3e-92	99%	LC032040.1

Table 2: Sequences producing significant alignment National Center for Biotechnology Information (NCBI) blast.

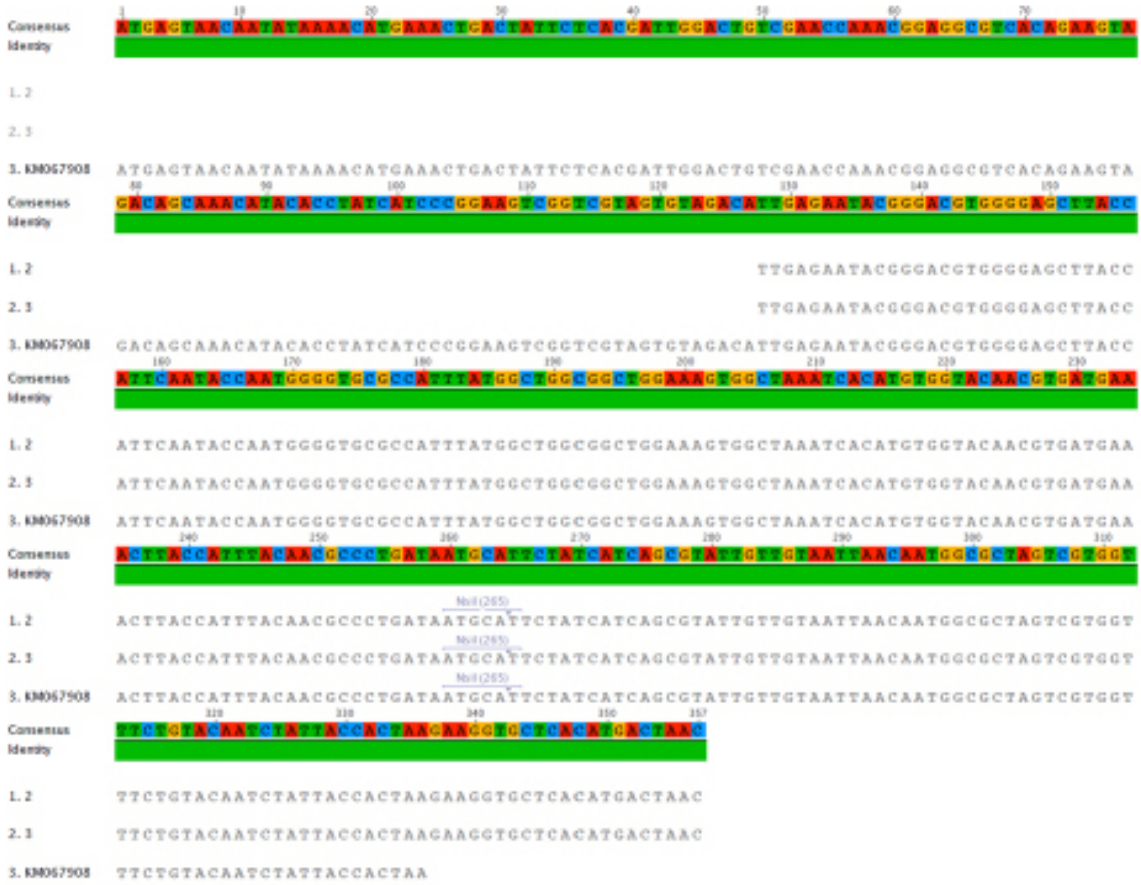

Figure 4: Result of BLAST and alignment analysis.

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The results of BLAST and alignment analysis from National Center for Biotechnology Information (NCBI) showed that the sample was identical to accession number KM067908.1 (Figure 3) that is *Vibrio parahaemolyticus* 13-028/A3 and 13-028/A2. Strain 13-028/A3 was determined to cause this disease through laboratory bioassays (Table 3).[7]

Mortality			
Duncan[a]			
		Subset for alpha=.05	
Treatment	N	1	2
Treatment Feed Group	6	.0000	
Negative Control Group	6	.0000	
Positive Control Group	6		46.6667
Sig.		1.000	1.000

Means for groups in homogeneous subset are displayed.
[a]Uses Harmoni Mean Sample Size = 6.000.

Table 3: Statistical analysis of AHPND challanged treatment and control groups using ANOVA.

Statistical Analysis

There was significant difference between treatment and positive control in terms of cumulative mortality.

DISCUSSION

The AHPND has appeared in almost all major shrimp producing countries of South East Asia. Much effort has been made to minimize the effect of disease by application of various available products like, probiotics, bacteriophages, immunostimulants, herbal extracts, quorum quenching, acidifiers and toxin absorbents, etc., with potential of anti-AHPND properties. However, most of them could not achieve the successful outcome as per expectation. The combinations of 10 natural oils were formulated to develop as anti-AHPND in the present study. The selection of oil for the formulation was done on the basis of their anti-viral and anti-bacterial properties. The product is combination of blend essential oils. The artificial feed as a carrier of anti-AHPND product is one of the best ways to provide the protection to the shrimp. Vietnam is one of the major suffering countries due to AHPND and so was selected for the trial site.

The final laboratory results provide conclusive evidence that in a controlled environment anti-AHPND feed provide prevention against AHPND-*Vibrio parahemolyticus*. The treatment shrimp that ingested AHPND infected tissue did not develop AHPND and did not show gross signs and no mortality during the experimental period. However, in contrast the shrimps in the positive control group demonstrated up to 46.7% mortality with clear symptoms of AHPND. The AHPND gross-sign appearance in the challenged shrimp was similar and as described by NACA, OIE, Tran et al.[13,14,7] There was feed drop on first 3 days of challenge in positive control, which indicates that the digestive system of the challenged animals without protection was damaged. The immersion method of challenge using purified and certified pathogenic *Vibrio parahemolyticus* procured from University of Arizona was selected to avoid any risk of contamination. The rate of mortality in the challenged group was similar to the field reports i.e. heavy mortality for 3 to 5 days with significant drop in feed intake. The bioassay challenge trial conducted by Tran et al[7] received the similar rate of mortality as recorded in the positive control of the current trial. The *Vibrio parahemolyticus* has short generation time which facilitates colonization and result of which large number of cells can dominate the host in a short period of time, giving it advantage to dominate over other bacteria in the surrounding environment.[8,15]

The sequencing alignment analysis results showed that the isolated *V. parahemolyticus* causing typical symptoms of AHPND is 100% matching with the strain isolated by Tran et al and Lightner DV.[7,16] The core genome of all *V. parahemolyticus* strains is composed of 3,284 genes, 24.3% of total pangenome. The core genome of only the pathogenic strains has 3,358 genes (FAO 2016).[8] Whereas, there are possibilities of more than one strain of *V. parahemolyticus* or isolates of other *Vibrio* species carrying the pathogenic genes (PIR A and PIR B) to cause symptoms like AHPND in shrimp.[8,17,18]

CONCLUSION

The obtained results and the analysis shows that the shrimp fed on blended oil mixed with feed have significant protection against AHPND-*Vibrio parahemolyticus*. The next step would be to conduct field trials in culture ponds to determine the efficacy of developed feed against AHPND.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Brief Research

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Update on Urinary Tract Infections in Dogs in a Tropical Island and Antimicrobial Susceptibility of *Escherichia Coli* Isolates for the Period 2010-2016

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ABSTRACT

Background: Published information on bacterial urinary tract infection (UTI) in dogs in tropical countries, including the Caribbean is scanty. Monitoring antimicrobial resistance helps in empirical selection of drugs. The objectives of this study were to examine the diagnostic laboratory records on canine UTI in Grenada for the period of 2009 to 2016, and to identify drugs which are most likely to be effective.
Methods: Urine samples from 468 dogs suspected of urinary tract infection (UTI) were quantitatively cultured, and the bacterial isolates were identified by phenotypic methods. Antimicrobial susceptibility tests were done using the standard disk diffusion method against 6 or more drugs.
Results: Of the 150 culture-positive samples, 71.3% were positive for Gram-negative bacterial UTI, and *Escherichia coli* (*E. coli*) were the predominant species. Antimicrobial resistance was least to enrofloxacin (7.8%) and most to tetracycline (58.7%) when both Gram-negative and Gram-positive isolates were considered together. With regard to *E. coli*, none of the 39 isolates tested were resistant to imipenem, a last-resort drug, but 42% isolates showed resistance to 2 or more classes of drugs, including 2 isolates with multi-resistance to 6 classes of drugs. One *E. coli* isolate was resistant to 5 classes of drugs, including resistance to extended spectrum cephalosporins (ESC), as indicated by resistance to cefotaxime and 4 other cephalosporins.
Conclusions: This study confirms that *E. coli* is the common bacterial species associated with UTI in dogs in Grenada, and that resistance is minimal to enrofloxacin when all bacteria are considered together. However, emerging *E. coli* with multi-drug resistance to ESC is of concern, and continued monitoring is important for prudent use of antimicrobial drugs, and to detect emerging trends in veterinary medicine, including those of public health significance such as resistance to ESC, which may be transferred to human pathogens.

KEYWORDS: Urinary tract infection; Dogs; Grenada; Bacteria; *Escherichia coli*; Antibiotic Susceptibility.

ABBREVIATIONS: UTI: Urinary Tract Infection; ESC: Extended Spectrum Cephalosporins; *E. coli*: *Escherichia coli*.

INTRODUCTION

Urinary tract infections (UTI) are the most common cause of urinary tract disease in dogs. Approximately 14% of all dogs will acquire bacterial UTI during their lifetimes.[1] The most frequently isolated bacterium from dogs with UTI is *Escherichia coli (E. coli)*, followed by *Staphylococcus* spp., *Proteus* spp., *Streptococcu* spp., *Klebsiella* spp., *Enterobacter* spp., and

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Pseudomonas spp.[2,3] Suggested drugs for treatment of UTI include amoxicillin-clavulanate, trimethoprim-sulfa, cepahlexin, fluoroquinolones, tetracycline, and ampicillin. Alternative drugs include gentamicin and chloramphenicol.[3,4] Fluoroquinolones such as enrofloxacin are particularly effective in treating UTI due to the high drug concentrations achievable within the urinary tract.[5] In a study in the U.S, over 80% of urinary tract isolates were found to be susceptible to enrofloxacin.[5]

Despite several recent publications on bacterial UTI in dogs from Europe and the United States,[6-9] there is scanty information from tropical areas. The results of a study on the canine urine samples from cases suspected of UTI in Grenada, a tropical Caribbean island nation during the period of 2004 to 2009 were published recently by Hariharan et al.[10] *E. coli* was the predominant species associated with UTI, followed by *Proteus mirabilis* and *Staphylococcus intermedius/Staphylococcus pseudintermedius*. Among six antimicrobials tested against, resistance was least for enrofloxacin, and most for tetracycline. Monitoring changes in antimicrobial efficacy and microbial resistance guides the empirical use of antimicrobials for the treatment of UTI and helps formulate strategic plans to limit drug resistance.[6] The objectives of this study, therefore, were to examine the overall picture of canine UTI in Grenada for the period of 2010-2016, and to identify drugs which are most likely to be effective for empirical use.

MATERIALS AND METHODS

We conducted a retrospective study of all submissions of urine samples from dogs to the diagnostic bacteriology unit of the St. George's University School of Veterinary Medicine (SGU) for the last 6 years. The laboratory received specimens for routine culture and sensitivity testing from the veterinary clinics in Grenada, including the SGU Small Animal Hospital.

Information on sex, and age of each dog, and treatment administered, if any, and the method of urine collection were collected from the case histories. Urine samples were stored at 5 °C and quantitatively cultured on the same day, and results interpreted as recommended by Osborne.[1] Inoculations were done routinely on blood agar (Columbia agar with 5% sheep blood, Remel Inc., Lennexa, KS, USA) and MacConkey agar (Remel Inc., Lennexa, KS, USA), using a calibrated inoculation loop that delivers 1 µl as per the standard method.[1] After incubation at 37 °C for 18-24 hours, the plates were examined to determine if cultures were pure or mixed, and the colonies were counted. Cut-off values for significant bacteriuria were: ≥1000 colony forming units (CFU)/ ml for samples collected by cystocentesis, ≥10,000 CFU/ml for samples collected by catheterization, and ≥100,000 CFU/ml for voided urine.[2] Mixed growth of more than 2 colony types was considered as indicative of contamination. Bacterial isolates were identified by the standard phenotypic characteristics as outlined by Quinn et al.[11] In case of inconclusive results, API bacterial identification strips (Analytab Products, BioMérieux, Mary-lEtoile, France, Europe) were used. Antimicrobial susceptibility testing was performed by the Kirby-Bauer disk diffusion method, as outlined by Quinn et al.[11] on Mueller-Hinton (MH) agar. The zone sizes were interpreted as susceptible, intermediate, or resistant, based on the guidelines provided by the Clinical Laboratory Standards Institute (formerly, National Committee for Clinical Laboratory Institute/NCCLS).[12] An *E. coli* strain ATCC 25,922 (American Type Culture Collection), susceptible to all antibiotics, was used as a control.

RESULTS

Of a total of 468 urine samples cultured, 318 were negative for bacterial growth, or had only insignificant growth or mixed growth indicating contamination. Of 150 culture positive samples, indicating urinary tract infection, 109 were from female dogs and 41 from male dogs. The proportion of female dogs with positive bacterial UTI was significantly higher than that of the male dogs (χ^2=6.372, df=2, p=0.006). The ages of animals varied from 6 weeks to 16 years, and 90% of animals were above one year of age. Eighty seven percent of urine samples had been collected by cystocentesis. Of 23 dogs with a history of antibiotic use, 8 had a course of amoxicillin-clavulanic acid, 2 enrofloxacin, and 1 ciprofloxacin prior to sample submission. The remaining were on doxycline, amoxicillin, ampicillin-sulbactam, cephalexin, or cefazolin.

Of one hundred and fifty culture-positive samples, none had mixed bacterial infection with 2 species. Table 1 lists the bacteria isolated from the 150 culture-positive samples, and the percentages of various genera and species. Of the 150 isolates, 71.3% were Gram-negative bacteria; the most frequent one was *E. coli* (28.7%), followed by *Proteus mirabilis* (20%), *Klebsiella pneumoniae* (6%) and *Pseudomonas aeruginosa* (4.7%). Among Gram-positive isolates (28.7% of total), the most common species was *Staphylococcus intermedius/Staphylococcus pseudintermedius* (7.3%).

The rates of resistance against 6 antibiotics, that are used to treat UTI in dogs[3,4,13] are presented in Table 2. Resistance was least (7.8%) to enrofloxacin, when both Gram-negatives and Gram-positives were considered together. However, when Gram-positives alone were taken into account, resistance was least (4.7%) to amoxicillin-clavulanic acid. Resistance was highest to tetracycline, the rate being 61.7% for Gram-negatives, and 50% for Gram-positives.

With regard to *E. coli*, which was the commonest species, none of the isolates were resistant to imipenem (Table 3). Among the other drugs, least resistance was seen to ceftriaxone (4%), and most to cephalothin (71.4%). Of 16 isolates tested against cefotaxime, one was found resistant. Eighteen (42%) of 43 isolates showed resistance to 2 or more classes of drugs. Two isolates showed simultaneous resistance to 6 classes of drugs: An aminoglycoside (gentamicin), beta-lactam drugs (ampicillin, amoxicillin-clavulanic acid, cephalothin), chloramphenicol, quinolones (ciprofloxacin, enrofloxacin), trimethoprim-sulfon-

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Table 1: Bacterial Species and the Number of Isolates from 150 Culture-Positive Urine Samples of a Total of 468 Samples Tested from Dogs in Grenada 2010-2016.

Bacterial species	Isolates # (%)	Gram stain id # (%)
Escherichia coli	43 (28.7)	Gram negatives: 107 (71.3) isolates
Proteus mirabilis	30 (20)	
Proteus vulgaris	8 (5.3)	
Klebsiella pneumoniae	9 (6)	
Pseudomonas aeruginosa	7 (4.7)	
Enterobacter cloacae	5 (3.3)	
Enterobacter aerogenes	2 (1.3)	
Pasteurella spp	1 (0.7)	
Serratia marcescens	1 (0.7)	
Serratia rubidaea	1 (0.7)	
Staphylococcus aureus	7 (4.7)	Gram positives: 43 (28.7) isolates
Staphylococcus intermedius	11 (7.3)	
Staphylococcus spp	10 (6.7)	
Streptococcus canis	1 (0.7)	
Streptococcus uberis	1 (0.7)	
Streptococcus spp	9 (6)	
Aerococcus viridans	3 (2)	
Enterococcus faecalis	1 (0.7)	
Total	**150**	

Table 2: Antimicrobial Resistance of Bacteria Isolated from UTI in Dogs in Grenada 2010-2016.

Antimicrobials	Resistant Gram-negative isolates/number tested	Resistant Gram-positive isolates/number tested	Total Resistant isolates/ total number tested
Cephalothin	26/43 (60%)	4/17 (23.5%)	30/60 (50%)
Trimethoprim/sulfamethoxazole	13/74 (17.6%)	8/28 (28.6%)	21/102 (20.6%)
Gentamicin	11/96 (11.5%)	3/42 (7.1%)	14/138 (10.2)
Tetracycline	29/47 (61.7%)	8/16 (50%)	37/63 (58.7)
Enrofloxacin	6/103 (5.8%)	5/38 (13.2%)	11/141 (7.8%)
Amoxicillin/clavulanic acid	28/90 (31.1%)	2/43 (4.7%)	30/133 (22.6)

amide, and tetracycline.

All *Proteus mirabilis* isolates tested were susceptible to ceftazidime, and gentamicin. Multiple resistance to 2 or more classes of drugs was seen among 47% of *P. mirabilis*. One of 16 isolates of *E. coli* and 2 of 14 isolates of *P. mirabilis* showed resistance to cefotaxime. All *Pseudomonas aeruginosa* isolates were susceptible to ceftazidime, ciprofloxacin, enrofloxacin, gentamicin, and imipenem, but resistant to tetracyclines. All *Staphylococcus intermedius* isolates were susceptible to amoxicillin-clavulanic acid and enrofloxacin.

DISCUSSION

Of 468 samples, 150 (32.1%) were positive for significant growth in culture, indicative of bacterial UTI. This is somewhat similar to a recent report from New Zealand where 37.7% of urine samples were positive in culture.[14] All 150 culture-positive samples in the present study had growth of a single bacterial species in high numbers indicative of UTI, and *E. coli* was the most common isolate. These findings are in agreement with the observation that UTIs in dogs are generally caused by a single bacterial species, the predominant being *E. coli*.[15] Several recent studies on UTI in dogs in temperate countries[6-9] have shown that *E. coli* is the predominant cause of UTI as seen in the present study and our previous study for the period of 2004-2009 in Grenada.[10] UTI is more common in female dogs, which is in agreement with the results of the previous study in Grenada. In contrast to the results of a study on bacteria from UTI in dogs in Sweden,[7] drug resistance is high in the dogs in Grenada. Antimicrobial resistance in *E. coli* is of concern in this tropical island, except for imipenem, which is a last-resort drug for multidrug-

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Table 3: Antimicrobial Resistance* of *Escherichia Coli* Isolates from UTI in Dogs in Grenada: 2010-2016.

Antimicrobial drugs	Number tested	Resistant	Susceptible	Intermediate
		Number (%)		
Ampicillin (AM)	18	10 (55.5)	5 (27.8)	3 (16.7)
Doxycycline	20	7 (35)	11 (55)	2 (10)
Cephalothin (CF)	21	15 (71.4)	1 (4.8)	5 (23.8)
Trimethoprim/sulfamethoxazole (TMS)	30	6 (20)	22 (73.3)	2 (6.7)
Gentamicin (GE)	36	10 (27.8)	26 (72.2)	0
Tetracycline (TE)	20	10 (50)	10 (50)	0
Chloramphenicol (CL)	15	4 (26.7)	10 (66.7)	1 (6.7)
Enrofloxacin (ENR)	41	3 (7.1)	35 (85.4)	3 (7.3)
Imipenem	39	0	39 (100)	0
Amoxicillin/clavulanic acid (AMC)	32	11 (34.4)	18 (56.3)	3 (9.4)
Ciprofloxacin (CIP)	42	3 (7.1)	39 (92.9)	0
Ceftazidime (CFZ)	38	3 (7.9)	34 (89.5)	1 (2.6)
Cefotaxime (CFT)	16	1 (6.3)	15 (93.8)	0
Cefpodoxime (CFP)	30	5 (16.7)	24 (80)	1 (3.3)
Ceftriaxone (CFR)	25	1 (4)	23 (92)	1 (4)
Cephalexin (CFL)	17	8 (47)	2 (11.8)	7 (41.2)

*Multiple resistance to 6 classes of drugs (AM, AMC, CF, CIP, CL, ENR, GE, TE, TMS) was seen in 2 isolates. One isolate was resistant to cefotaxime with a resistance pattern: AM, AMC, CF, CFL, CFP, CFR, CFT, GE, TE, TMS.

resistant *E. coli*.[16] Over 40% of the *E. coli* isolates in this study showed multiple drug resistance to 2 to 6 classes of drugs, and one isolate was resistant to cefotaxime, which indicates resistance to extended spectrum cephalosporins.[7] This is of concern in small animal medicine as well as of public health significance when the genes are transferable. Overall, enrofloxacin still remains the most effective drug against urinary pathogens from dogs in this country, more than 92% of all isolates being susceptible. This finding is in agreement with that of a study done in the United States.[5] Fluoroquinolones are among the few drugs of choice for treatment of bacterial urinary tract infections in dogs, including those due to *Pseudomonas aeruginosa*.[13] The disk diffusion method underestimates the effectiveness of some drugs, because it is based on expected serum concentrations. Several drugs, including enrofloxacin reach much higher concentrations in the urine.[4] Although, over half the isolates were resistant to tetracycline, concentrations of doxycycline attained in the urine of dogs can inhibit majority of urinary pathogens.[17] In any case, urine culture remains the "gold standard" for diagnosis of urinary tract infection, and culture and susceptibility testing should particularly be performed in cases that do not respond within 7 days of therapy.[3] Prior to obtaining culture and sensitivity results, Gram's stain may allow identification of the pathogen as Gram-positive or Gram-negative, and this will be helpful in determining initial therapy.[13]

In conclusion, this retrospective study on urinary pathogens from dogs for the period 2010-2016 confirms the results of our previous findings for 2004-2009 that *E. coli* is the most common bacterial species, and resistance to enrofloxacin is minimal. However, emerging multi-drug resistance such as resistance to cefotaxime, which is generally associated with the production of extended-spectrum beta-lactamases[7] is of concern. Prudent use of antimicrobial drugs, and continued monitoring of trends in drug resistance are important in controlling bacterial resistance to antibiotics.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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Research

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Hazard Assessments of Cattle Fascioliasis With Special Reference to Hemato-Biochemical Biomarkers

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ABSTRACT

Aim: Fascioliasis is a well known veterinary trouble in cattle and is to date an important human being disease. The aim of this study was to evaluate the functional capacity of the liver based on the activity of specific enzymes, proteins and glucose in serum and also to investigate the influence of mechanical and toxic effects of fascioliasis on blood picture and biliary tract of cattles.
Materials and Methods: This study was carried out between January and April 2016. Blood and fecal samples were collected from 22 cattles with naturally acquired bovine fascioliasis and no other disease out of 150 cattles and 10 non-infected cattles were brought to the Veterinary Clinic, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt. Blood was drawn from all the examined animals to measure the respective levels of hematological and biochemical parameters.
Results: Investigation of the animals infected with fascioliasis showed paleness of mucous membranes, loss in weight, watery diarrhea, visible submandibular edema and normal rectal temperatures. Normocytic normochromic anemia with a significant decrease ($p<0.05$) in red blood cells count (RBCs), hemoglobin (Hb) concentration, percentage of packed cell volume (PCV %) were observed in an infected cattle compared to the uninfected control. In addition, leukocytosis, neutrophilia, eosinophilia, lymphopenia and monocytopenia were reported. Moreover, significant changes in the biochemical parameters were recorded.
Conclusion: From this study, it could be concluded that the determination of hematological and biochemical parameters are useful in early diagnosis and prognosis of cattle fascioliasis.

KEY WORDS: Hemoglobin; Leukocytes; Liver enzymes; Albumin; Creatinine.

ABBREVIATIONS: RBCs: Red Blood Cells count; Hb: Hemoglobin; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl tranferase; WBCs: White Blood Cells.

INTRODUCTION

Fasciolosis is a systemic parasitic disease of cattle caused by the liver flukes, *Fasciola gigantica* and *Fasciola hepatica* (trematodes of the genus *Fasciola*). Fascioliasis merits particular consideration due to its global distribution in livestock and the estimation of up to 17 million people infected worldwide. It is an important hepatic disease causing great enormous economic losses in ruminants and in livestock industries through animal mortality, growth retardation, sterility, condemnation of affected livers and expense due to control measures.[1,2]

Apart from its high veterinary importance, fasciolosis is a secondary zoonotic infection of a good number of human populations in every continent of the world. The increasing importance of human fascioliasis is also related to its pathogenicity and immunity. This disease is pronouncedly complicated, including difficulties in diagnosis, its great morbidity

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and immunological impact on children in long-term infection particularly in human fascioliasis endemic areas.[3,4]

Fascioliasis has the widest geographic spread of any vector-borne zoonotic disease occurring in more than 51 countries worldwide. In the Islamic Republic of Iran, Western Europe, Cuba, Egypt, and the Andean countries of South America about 91 million people are at a risk of getting infected.[5]

Since the liver is the main metabolic organ of the body, infection of the hepatocytes is an essential feature of certain parasitic infections. Due to the complexity of vascular and biliary system examination, there are a number of different laboratory tests, which are based on biochemical analysis of serum parameters. These tests usually include the determination of serum transaminases alanine transaminase, aspartate transaminase (ALT, AST), which are the most sensitive indicators of hepatocellular injury. Alkaline phosphatase (ALP), gamma-glutamyl tranferase (GGT), serum proteins and bilirubin are also used to evaluate the degree of cholestasis and synthetic capacity of the liver.[6] In fascioliasis, the metabolic processes of the liver[7] and kidney are gradually reduced.[8]

Chronic fascioliasis is accompanied by weight loss, weakening, pallor of mucous membranes, anemia, ventral edema, appetite changes, soft stool consistency or diarrhea, stomach hypotonia and decreased milk production.[9] Clinical diagnosis and positive fecal egg counts along with elevated level of gamma-glutamyl transferase (GGT) confirm for the presence of chronic fascioliasis. The diagnosis of acute fascioliasis is implemented by the determination of serum hepatic enzyme activities which are released from the damaged hepatic cells.[10]

The aim of this study was to diagnose the functional capacity of the liver based on the activity of specific enzymes, proteins and glucose in serum and also to investigate the influence of mechanical and toxic effects of fascioliasis on blood picture and biliary tract of cattles.

MATERIALS AND METHODS

Ethical Approval

All study procedures were approved by and in accordance with the rules of animal use and care by the ethical committee of Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt.

Animals and Samples

This study was carried out between the months of January and August in the year 2016. A total of 150 animals were admitted to the Veterinary Clinic, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt because of altered appetite, weight loss and diarrhea in some cases. These animals were aged between 2 to 3 years and weighed between 350 to 450 kg. Fecal and blood samples were collected from 22 cattles with naturally acquired bovine fascioliasis and no other disease out of 150 cattles and 10 non-infected cattles. The selected cattles for study were confirmed to be free of other possible diseases.

Fecal samples were collected from the rectum for fecal egg counts. Fecal samples were examined by direct smear, floatation and sedimentation techniques for the presence of fluke eggs according to Urquhart et al.[11] Two blood samples were taken from the jugular vein. The first sample was collected in evacuated Ethylenediaminetetraacetic acid (EDTA) tubes and used for hematological studies. The second was drawn into clean and dry serum separating tubes, left to clot and later centrifuged for 20 minutes at 2000 rpm for serum separation. Clear serum samples were carefully aspirated and stored at -20 °C to be used for biochemical analysis.

Hematological Studies

Total white blood cells (WBCs), red blood cells (RBCs), hemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated using an automated digital blood cell counter (Hospitex-Hemascreen 18, Fiorentino, Italy) using the veterinary software. Differential blood counts were determined using Giemsa-stained blood smears.

Biochemical Studies

Serum activities of aspartate aminotransferase (AST) and gamma glutamyl transferase (GGT) and serum concentrations of glucose, total protein (TP), albumin, blood urea nitrogen (BUN), and creatinine were determined according to standard procedures using commercial kits (Diamond, UK). Serum enzyme activities were determined at 37 °C. The concentration of globulins was calculated.

Statistical Analysis of Data

The data obtained was analyzed using SPSS version 16. The student *t*-test was used to analyze the significant differences between the hematological and biochemical parameters of the *Fasciola*-infected and the non-infected samples. Values of $p<0.05$ were considered significant.

RESULTS

Clinical Findings

The infected animals showed prominent clinical signs of progressive weight loss and watery diarrhea. The mucous membranes were pale, with visible submandibular edema in some infected cases, and the rectal temperatures were all within normal range. Fecal examination revealed yellow brown thin walled, operculated eggs specific for *fasciola* species (Figure 1).

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Figure 1: Fasciola Eggs (X100).

Adult flukes of both species (*Fasciola hepatica* and *Fasciola gigantica*) are localized in the bile ducts of the liver or gall bladder. *Fasciola gigantica* measures 4 to 10 cm in length. *Fasciola hepatica* measures 2 cm to 3 cm in length and 1.3 cm in width, and has a cosmopolitan distribution. *Fasciola hepatica* is one of the largest flukes in the world. The adult worm has a characteristic leaf shape with the anterior end being broader than the posterior end and anterior cone and a ventral sucker at the base of the cone which allow it to attach to the lining of the biliary ducts. Each worm possesses ovaries and testes which are highly branched and allow for individual flukes to produce eggs independently.

Hematological Findings

The hematological results as shown in Table 1, indicated that RBCs counts, PCV, and Hb concentration were significantly ($p<0.05$) decreased in the infected cattle than in the uninfected control. No significant differences occurred between the erythrocytic indices (MCV, MCH & MCHC) of the infected and non-infected cattle indicating normocytic normochromic anemia. The results of leukogram of the infected and uninfected cattle are presented in Table 2. There was a significant ($p<0.05$) increase in the total leucocytes, neutrophils and eosinophils of the infected cattle with monocytopenia and lymphocytopenia in

Table 1. The Mean Values of the Erythrogram of the Uninfected and *Fasciola*-Infected Cattle (Mean±SE).

Parameters	Uninfected group n=10	Infected group n=22
RBCs ($x10^6$)	7.40±0.33	5.00±0.11*
PCV (%)	38.15±0. 90	25.50±0.55*
Hb (g/dl)	12.59±0.15	8.5±0.14*
MCV (fl)	51.55±0.94	51.00±0.43
MCH (pg)	17. 01±0.38	17.00±0.35
MCHC (%)	33.00±0.52	33.33±0.58

Statistical significance between the infected group and infected group; *$p<0.05$

Table 2: The Mean Values of the Total and Differential Leucocytic Counts of the Uninfected and *Fasciola*-Infected Cattle (Mean±SE).

Parameters	Uninfected group n=10	Infected group n=22
WBCs ($x10^3$)	9.51±4. 67	14. 59±5.74
Neutrophils ($x10^3$)	3.45±1.02	7.85±1.27*
Eosinophils ($x10^3$)	0.70±0.49	3.00±0.40*
Basophils ($x10^3$)	00	00
Monocytes ($x10^3$)	1.11±0.50	0.59±0.17*
Lymphocytes ($x10^3$)	4.25±0.86	3.15±0.61*

Statistical significance between the infected group and infected group; *$p<0.05$

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Table 3: Biochemical Parameters of the Uninfected and *Fasciola*-Infected Cattle (Mean values±SE).

Parameter	Uninfected group n=10	Infected group n=22
AST (U/l)	44.35±4.55	83.00±6.67**
GGT (U/l)	66.25±5.81	93.70±9.00**
Total protein (g/dl)	7.55±1.24	5.97±0.62*
Albumin (g/dl)	3.53±1.09	1.10±0.76*
Globulins (g/dl)	4.02±15.40	4.87±5.45*
Glucose (mg/dl)	55.07±1.17	40.16±3.27*
BUN (mg/dl)	45.18±1.64	70.52±2.63**
Creatinine (mg/dl)	1.21±0.51	1.95±0.18**

Statistical significance between the infected group and infected group; *$p<0.05$, **$p<0.01$

the infected cattle. No difference was observed in the values of basophils in both groups.

Biochemical Findings

The biochemical results (Table 3) revealed that the infected animals with fascioliasis had a significant ($p<0.01$) higher activities of serum AST and GGT with a significant ($p<0.05$) lower serum glucose, total proteins and albumin levels, as well as higher globulins levels than in the non-infected group. Moreover, the serum urea and creatinine levels were significantly ($p<0.01$) increased in the infected animals.

DISCUSSION

The progressive weight loss and pale mucous membranes in infected cattles may be attributed to anemia caused by Fascioliasis. Bottle jaw syndrome could be as a result of massive *Fasciola* infection causing liver destruction leading to cessation of protein synthesis.[12,13] The distribution of the *Fasciola* species is limited to the tropics and has been recorded in South and Eastern Asia as well as in the Middle East, Eastern Europe and Africa.[14] The clinical examination alone was not enough to diagnose the underlying pathogen; therefore, it should be used together with other laboratory diagnostic methods. The hematological results revealed normocytic normochromic anemia with decreased RBCs counts, PCV, and Hb concentration in the infected cattle may be attributed to chronic blood loss due to the blood-sucking activity of the adult flukes and leakage of blood from the bile duct to the intestine. The decrease in RBC counts can be due to decreased production or peripheral destruction of RBCs or blood loss from direct blood feeding by the flukes: Blood has been recovered from regurgitated caecal contents and from hemorrhage into the parenchyma, the bile ducts and the abdominal cavity as a result of activity of the fluke.[9,15,16] The decrease in Hb concentrations may be due to deficiencies in iron implicated in fasciolosis and is a factor interfering with the synthesis of hemoglobin. The reduction in RBC counts, Hb and PCV in bovine fascioliasis may attributed to extensive loss of blood into bile duct due to the large amounts of flukes present in the liver or the acute loss of blood caused by the flukes.[2,16] The severe anemia may be attributed to a chronic liver inflammation, which causes erythrogenesis depression.[17-19] Similar results for RBC and PCV were observed in cattle,[20] while the results for RBC and Hb were similar to those reported in cattle[21] and in sheep.[7,22,23] Moreover, the same observations in RBC, Hb and PCV were noticed in sheep with fascioliasis.[10,24] This reduction was also found to be inversely proportional to worm load, which is in agreement with the previous findings,[25] who reported that there is a good correlation between the level of infestation and RBC numbers, PCV, Hb content and plasma protein values.

Leucocytosis, neutrophilia and eosinophilia with monocytopenia and lymphocytopenia were reported in all infected cattles. These results were consistent with those obtained in sheep[22] and bovine.[2] The changes in the differential counts may be a means of body defense against *Fasciola*, obstructive effects or due to the toxin mediated lesion of the bone marrow.[26] The neutrophilia and eosinophilia were also observed to be proportional to worm load. A positive correlation between eosinophil counts and the number of liver flukes recovered were previously observed.[27] This may be due to inflammation and infection resulting from the activity of the flukes in the bile ducts.[28] There are 3 main ways in which eosinophilia acts on the parasite. The mechanism of cytotoxicity cell-mediated antibody-dependent, in this case, the linking of the eosinophils to the target covered by various immunoglobulins; eosinophils may be linked with the targets covered by the C3b fragment of complement, exacerbating binding functions, causing damages to the parasite membrane; the interaction between immunoglobulin E (IgE) and eosinophils to obtain specific mediators, resulting in parasitic defense in two ways: local accumulation of neutrophils and other leukocytes, leukocyte activation and increasing their activity to damage parasites, and last would be action in the local tissue elements, intermediate local inflammation and producing unfavorable conditions for development of parasites. In addition, monocytopenia might be due to increased chemotaxis in response to the inflammatory process in the bile ducts.

With regards to the liver enzymes, the serum AST and GGT activities were significantly increased in the infected cattle comparatively with the uninfected. Liver enzymes activities

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are indicators of hepatic cell damage caused by fascioliasis.[24,29] *Fasciola hepatica* causes the release of reactive oxygen species causing cell wall damage and hepatic tissue necrosis.[30] These changes influence the biochemical parameters in the serum, and determination of specific liver enzymes functions as a valuable tool for diagnosis of hepato-biliary diseases. Increased values of serum AST could be related to the hepatocellular necrosis and degenerative changes produced by migration of immature flukes through the liver parenchyma. Moreover, the cellular changes from parasitism increase the permeability of the hepatic cells and in turn result in the release of the enzymes into the serum. Elevation in serum GGT levels was an indicator of chronic changes, cholestasis and epithelial damage in bile ducts caused by presence of adult flukes in biliary tract. These results agree with studies showing that a GGT increase provides sensitive indications of liver injury. GGT is the best marker, while AST is less sensitive.[24] Moreover, our findings were confirmed with those obtained previously in cattle and bovine,[31] cattle[19] and ruminants.[32] They reported degenerative changes in the hepatocyte and biliary cirrhosis in the liver histopathology of cows and cattle infected with *Fasciola*.

Hypoproteinaemia, hypoalbuminaemia and hyperglobulinaemia produced in this study were due to fascioliasis. This observation is in agreement with other studies in the cattle,[33] in fallow deer,[29] in sheep[10] and in bovine.[8] The hypoproteinaemia is due to severe infection of the liver produced destruction of liver parenchyma resulted in drastic alteration in protein values. The hypoalbuminaemia may be due to reduced albumin synthesis caused by liver damage. This produces cholangitis, biliary obstruction, destruction and fibrosis of hepatic tissue and anaemia.[28] The detected hyperglobulinaemia could be as a result of immune response to infection[10] and due to the increase in α and β globulin production.[34] The consequences of liver damage resulting from the migrating flukes compromises liver function which is reflected in changes of plasma proteins (albumin, globulins) concentration.[32]

The observed hypoglycemia may be attributed to the disturbance of glucogeneogenesis which results from hepatic disorders[35]; elevation of the ketone bodies during gastroenteritis which results in blood glucose depression[34] and depression of the hepatic glucogenic pathways and decrease in voluntary feed intake by the infected animals. Liver parenchymal damages inflicted by higher worm load could possibly be the cause of hypoglycemia.[8]

Elevations of serum urea and creatinine levels were due to *Fasciola* infection in cattle. These results may be due to renal problems. In agreement with others[36] who reported that glomerulopathy is associated with fascioliasis and buffaloes are suitable as a naturally existing experimental model of renal injury by circulating immune complexes.

CONCLUSION

It could be concluded that measuring the hematological and biochemical parameters could be useful in early diagnosis and prognosis of cattle fascioliasis. Additionally, changes in the levels of hepatic enzymes (AST and GGT) released into the blood as a result of liver tissue damage are used to monitor the progress of the infection and as a sensitive diagnostic aid in field infection.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interests.

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Case Report

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MR Signal Changes of Trigeminal Nuclei in a Case of Suspected Idiopathic Trigeminal Neuropathy in a Staffordshire Bull Terrier

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ABSTRACT

Trigeminal neuropathy, often idiopathic, is described as a common and self-limiting condition of dropped jaw in dogs. Previously described magnetic resonance findings are limited and describe thickening of the trigeminal nerve only. In this article, we report a Staffordshire bull terrier that presented with dropped jaw and was found to have bilateral hyper intensities at the location of the trigeminal nuclei following magnetic resonance imaging (MRI). Testing for infectious diseases and examining the cerebrospinal fluid (CSF) sample obtained from the cisterna magna did not identify an underlying pathology, and the dog proceeded to make a full clinical recovery following anti-inflammatory treatment. In the authors knowledge, this represents the first case reported with the presentation of trigeminal neuropathy and changes seen within the brain on MRI.

KEYWORDS: Trigeminal neuropathy; Magnetic resonance imaging (MRI); Dog.

ABBREVIATIONS: ITN: Idiopathic Trigeminal Neuropathy; MRI: Magnetic Resonance Imaging; CSF: Cerebrospinal fluid; EMG: Electromyography.

INTRODUCTION

Trigeminal neuropathy is described as the most common neurological cause of dropped jaw in dogs.[1] Also referred to as idiopathic trigeminal neuropathy (ITN), trigeminal neuritis, and trigeminal neurapraxia, this condition primarily affects the motor branches of the trigeminal nerves bilaterally, although associated loss of facial sensation and Horner's syndrome are described.[2] The condition is self-limiting and is characterised by spontaneous full recovery. The published literature on magnetic resonance imaging (MRI) findings for this condition is limited to two cases, and describes non-specific changes within the trigeminal nerve.[3] To the author's knowledge, this is the first reported case of suspected ITN with MR signal changes within the brain.

CASE HISTORY

An 8-year 9-month old male Staffordshire bull terrier presented to the neurology department of Dick White Referrals (Newmarket, UK) with a 4-day history of an inability to close the jaw, excessive salivation and difficulty eating and drinking. On neurological examination, there was a complete absence of jaw tone with normal visual and palpable temporal muscle mass. Facial sensation was normal, as was the gag reflex, and no evidence of Horner's syndrome was seen.

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Remaining cranial nerve tests including menace response, pupillary light reflex, dazzle reflex, corneal and palpebral reflexes and oculovestibular response tests were normal. Neuroanatomical localisation was to bilateral trigeminal nerve (motor branch) dysfunction. A complete blood count and routine biochemistry panel were unremarkable. MRI was undertaken to further pursue a diagnosis.

Magnetic resonance (MR) images were acquired with a 0.4T magnet (Hitachi Aperto, Japan). Imaging sequences include SAG FSE T2, TRANS FSE T2, TRANS T2 Flair, TRANS T2* GRE, TRANS SE T1 and post contrast SE T1 in 3 orthogonal planes. Diffusion weighted images were also acquired in the transverse plane.

On the T2 weighted images symmetrical, ill-defined hyperintensities within the pons at the level of the trigeminal nuclei were seen (Figure 1). These areas appeared isointense on T1W images. There was mild heterogeneous contrast enhancement of the trigeminal nerve and ganglion bilaterally (Figure 2), with no enhancement seen within the pons itself. No mass lesion or mass effect was seen with the remainder of the brain parenchyma unremarkable on all sequences. There was no evidence of muscle atrophy or signal changes within the masticatory muscles. Regional lymph nodes appeared normal.

RT-PCR on cerebrospinal fluid (CSF) for *Distemper, Toxoplasma, Neospora, Bornavirus, Borrelia, Ehrlichia, Leishmania, Canine Herpes, Parvovirus* and *Minute* virus were negative. Given the bilaterally symmetrical changes and in absence of mass effect neoplasia was considered less likely and a presumptive diagnosis of inflammatory or idiopathic trigeminal neuropathy was made. A CSF sample obtained from the cistern magnum after MR imaging showed albuminolocytologic dissociation, with protein 0.41 g/L (ref: <0.30).

The dog was hospitalised on fluid maintenance and supportive feeding. Treatment was started with 0.2 mg/kg dexamethasone (Dexadreson; MSD Animal Health) intravenously and 4 mg/kg gabapentin (Gabapentin; Zentiva) per os every 24 hours, 2 mg/kg ranitidine (Zantac; GlaxoSmithKline) every 12 hours, and 90 mg/m^2 lomustine (Lomustine; medac). The dog began to persistently regurgitate for the 1st 24 hours in hospital. A single left lateral thoracic radiograph was unremarkable with no evidence of megaoesophagus. The regurgitation fully resolved on 1 mg/kg maropitant (Cerenia; Zoetis) every 24 hours and 1 mg/kg/24 hours metoclopramide (Emeprid; Ceva) continuous rate infusion.

Over 6 days of hospitalisation, the gabapentin dose was increased to 4 mg/kg every eight hours. Marginal improvement in the jaw tone was noted, and the dog was coping well with assisted feeding, drinking and per os medication. He was discharged to continue with 0.08 mg/kg dexamethasone every 24 hours and 4 mg/kg gabapentin every eight hours, with a 14 days course of 2 mg/kg ranitidine every 12 hours and 0.4 mg/kg metoclopramide every 12 hours.

At follow-up with the owner 2 weeks later by phone call he was continuing to do well, with gradually returning function and strength of the jaw. The owners reported he still required assistance to eat and drink but was improving with significantly reduced hypersalivation. The owners had noticed significant atrophy of the masseter muscles bilaterally, however, he had only recently returned to chewing food. At this point, the dexamethasone was reduced to 0.04 mg/kg every 24 hours and all other medications stopped. At a further phone call 2 months after discharge, the owners reported that gradual improvement had continued and he had recently become clinically normal in their opinion. All medications had finished 2 weeks prior to this check-up, and no further check-ups were advised unless signs

Figure 1: Transverse T2 Weighted FLAIR Image at the Level of the Pons. Bilateral Hyperintensities (White Arrows) are seen in the Regions of the Trigeminal Nuclei.

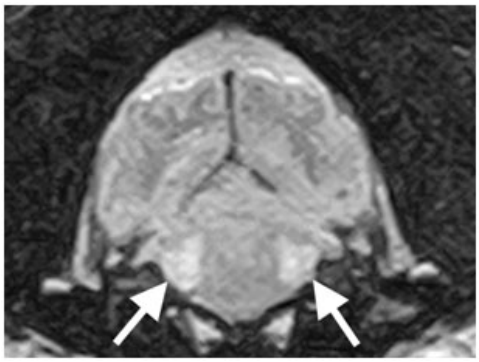
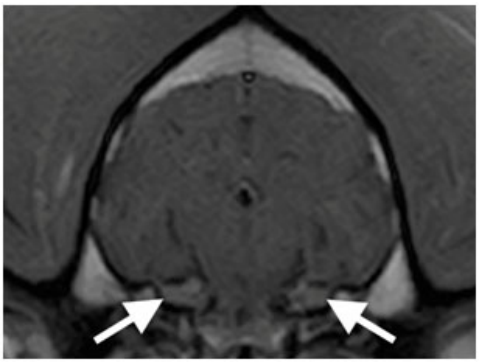


Figure 2: Transverse T1 Weighted Post-Contrast Image of the Brain Showing Normal Appearance and Enhancement of the Trigeminal Nerves at the Level of the Ganglia (White Arrows).



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of recurrence were seen. A final phone update 8 months after discharge confirmed no deterioration with normal jaw function.

DISCUSSION

Flaccid mandibular paralysis or 'dropped jaw' is caused by dysfunction in the motor branch of the trigeminal nerve, with potential aetiologies in dogs including infectious, inflammatory, neoplastic and idiopathic.[1] ITN is primarily a dysfunction of the motor tracts with associated 'dropped jaw'.[2] A minority of cases have facial sensory deficits and/or unilateral Horner's syndrome, although absence of concurrent neurological signs is common.[4] When cases of ITN were reviewed, the only tests to show abnormal results in most cases were CSF analysis and electromyography (EMG).[2] On CSF analysis, mild to moderate elevations in total protein, and mild elevations in nucleated cell counts, were the most common findings, consistent with the mild elevation in protein levels seen in this case.

A suspected diagnosis of trigeminal neuritis is confirmed when the clinical signs self resolve, usually within 2-3 weeks, although this has been reported to range from 4 to 63 days.[1,4] Interestingly, the use of steroid therapy has showed no significant difference in resolution time.[2] Given this pattern of spontaneous full clinical recovery, there is no definitive histological evidence of the disease from post-mortem, and therefore a poor understanding of its aetiology, although an inflammatory cause is suspected.

Reported findings on MR imaging of ITN are very limited. Given the resolving nature of the clinical signs, MR is often not undertaken when a diagnosis of ITN is suspected, it's main use has been to rule out neoplasia in cases of unilateral dysfunction, or where clinical signs progress or fail to resolve.[5,6] In two dogs with histological confirmation of neuritis, both had diffuse enlargement of the trigeminal nerves described within the calvarium and trigeminal canal, but with no discrete mass lesion or mass effect seen.[3] The nerves were isointense on T1W precontrast and PDW images in both dogs, with the nerves hyperintense on T2W images in one case and isointense on T2W images in the other. Either heterogeneous or homogeneous enhancement of the nerve was seen on post-contrast T1W images. Post-contrast enhancement of the ganglia was seen in this case however, enhancement of the trigeminal ganglia with or without enhancement of the associated nerves has been shown to be a normal finding.[6] In cases of nerve-sheath neoplasia, unilateral discrete contrasting enhancing mass lesions are described, with or without displacement of the adjacent brainstem.[3,7,8] No cases of idiopathic trigeminal neuropathy with MR changes within the brainstem, were found on review of the literature.

CONCLUSION

Giving the lack of histological confirmation for diagnosis, a broad treatment protocol was used. The dog progressed to full clinical improvement and remained so on withdrawal of all medications. This clinical behaviour is not consistent with the other considered differentials, and therefore this case is presented as a likely severe case of idiopathic trigeminal neuropathy with novel intra-cranial findings on MR.

CONFLICTS OF INTEREST

The authors declare they have no conflicts of interest.

CONSENT

The owner has given permission for publication of any material related to this case.

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Research

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The Social Behaviour of Neutered Male Dogs Compared to Intact Dogs (*Canis lupus familiaris*): Video Analyses, Questionnaires and Case Studies

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ABSTRACT

Introduction: Gonadectomy is a regularly performed procedure in domestic dogs. Apart from preventing reproduction, neutering is also conducted for reasons of health and to change or remove undesirable behaviour. From the ethological perspective, castration may have a negative impact on the social behaviour of male dogs.

Objectives: The question to be answered is whether there are differences in certain behaviours between neutered and intact male dogs and to gain further insight into the effects of neutering on a male dog's behaviour by comparing the social behaviour of neutered and intact animals in an observational part as well as by evaluating questionnaires submitted by dog owners.

Methods: Using video recordings from Germany and Switzerland of six groups of dogs, consisting of 18 intact and 16 neutered males in total, the social behaviour of the dogs was analyzed. Specific behaviours were compared between the castrated and intact males and statistically analyzed. Furthermore, 29 questionnaires based on the studies conducted by Turcsán et al on 104 dogs and their dog owners have been incorporated into this study. Additionally, 54 case studies from our consultancy were evaluated.

Results: Significant differences were found in patterns of behaviour such as smelling and licking the genital area, chin rest, tooth chatter and molesting other dogs (Mann-Whitney-U-Test, U=382.000, p=0.001, when analysed in frequencies; Randomization test for 'molesting', F=13.044; p=0.001, when analysed for its duration). Also, the non-castrated males show more behaviour indicating high status. The results of the questionnaires indicate a trend that neutered males react emotionally more unstable in stressful situations and the case studies show a tendency for aggressive behaviour and fear to be more frequent in castrated dogs.

Conclusion: Our results support the assumption that castration may have a negative influence on the behaviour of male dogs. Neutered dogs may gain attractiveness for intact dogs which can lead to a high stress factor for the castrates. Accordingly, dog owners should be aware of the consequences for the dog.

KEY WORDS: Neutering; Male dogs; Social behaviour; Video recordings; Questionnaire study; Case studies.

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INTRODUCTION

Neutering is seen as the primary method for population control and is also routinely conducted by animal shelters to avoid further multiplication of homeless dogs.[1,2] There are always controversial discussions about neutering dogs, since such an intervention implicates both advantages and disadvantages. Beside several health effects such as an increased risk for cancer, many behavioural changes such as excessive barking and increased anxiety can occur, as shown in a study of castrated Vizslas.[3] Thereby the age at the time of neutering, the breed itself and the sex of the animal are taken into consideration. Accordingly, these effects should be weighed against each other before the decision for or against a castration is taken.[4]

Neutering, gonadectomy or castration refers to the surgical removal of the reproductive organs, and consequently to a great decrease in the production of gonadal steroids.[5] Even though it is frequently recommended and performed however, the full extent of its consequences for the animal remains unclear. Over the last few years, some attention has therefore been given to the long-term health effects of neutering dogs. One repeatedly investigated issue is the influence of neutering on various types of cancer. On the one hand, there is a well-established opinion that the castration of bitches reduces their risk of developing mammary tumours. However, a recent review of this topic by Beauvais et al[6] states that currently, the evidence presented in the reviewed papers is insufficient to show a reductive effect of neutering on mammary tumours. On the other hand, an increased risk of various cancers has been shown in several studies. Teske et al[7] found that castration favours the progression of prostate carcinoma in dogs, and Zink et al[3] state that gonadectomised Vizlas have a higher probability of developing hemangiosarcoma, mast cell tumours, lymphosarcoma and lymphoma as well as other kinds of cancer. A study on Golden retrievers[2] as well as one reviewing the cause of death for over 40,000 neutered and intact dogs[8] present similar results, stating that neutered dogs have an increased risk for several types of cancer. Apart from cancer, neutering has also been shown to correlate with an increased rate of autoimmune diseases and other diseases such as joint disorders (hip dysplasia and cranial cruciate ligament tear;[2,9] and dermatological conditions such as a change in coat or fur quality).[10]

Adult male dogs are often castrated with the aim to remove undesirable behaviour such as mounting, roaming, aggressiveness and urine marking. However, the degree to which a behavioural pattern is changed *via* castration is not clearly defined.[11] On no account, can neutering replace a proper education and appropriate socialization of the dog. Nor should it be considered as a panacea against all undesirable behaviours, although it may have positive effects on the dog's behaviour in certain cases.[12] The effects of a castration are dependent on the dog's personality and they do not affect every behavioural pattern, since not all behaviours depend on sex hormones.

The experiences of the dog before neutering should not be disregarded. A dog can occasionally recall its possible positive sexual activities and will continue to show these behaviours, even though they might not be hormonally dependent.[5] Nevertheless, all animals should be considered individually with the supposition that the population control should play a subordinate role in relation to the health of the animals.[13]

Less well understood and perhaps more difficult to investigate are the effects of neutering on the behaviour of dogs. Research in this field has resulted in a better understanding of certain aspects of behavioural alterations in neutered dogs. For one, several studies show neutered dogs to be more excitable and anxious than their intact conspecifics: A review of medical records and online surveys found that neutered dogs are more likely to have separation anxiety and fear of storms than intact animals.[3,14] Additionally, an experimental study using 38 test subjects separated into three groups (gonadectomised at the age of seven weeks, seven months or left intact) judged all neutered dogs to be generally more active and males gonadectomised at seven weeks to be more excitable than intact individuals.[15] Gonadectomy was also found to be correlated with a higher risk of developing other behavioural problems connected to fear and anxiety as well as hyperactivity.[3] Concerning aggression, the public opinion of the effects of neutering seems to clash most severely with scientific evidence. There is a popular belief among dog owners that neutering result in less aggressive behaviour.[16] However, a study on aggression in English cocker spaniels found that neutered and intact males did not differ in their likelihood of showing aggressive behaviour in any of 13 tested situations.[17] Moreover, females neutered before showing any signs of aggression were more likely to behave aggressively towards children of the same household than intact females. Similar results of increased aggression in gonadectomised dogs were also found in more recent studies.[3,18] In addition to various behavioural aspects, neutering may also influence dogs on a cognitive level. This seems especially noticeable at a high age, when intact males show less or less severe signs of cognitive impairments than neutered males.[19] This is hypothesized to be an effect of the circulating testosterone, which may cause the progression of cognitive impairment to slow down. If and how the cognitive abilities of a dog are affected by neutering at a younger age is not yet known.[4]

Furthermore, there are studies in rodents, which indicate that estrogens improve memory and learning behaviour, for example by estradiol, which appears to interact with cholinergic systems. Both estrogens and testosterone reduce accumulation of the beta-amyloid, and testosterone, so it is suspected that it also prevents the phosphorylation of "neuroprotective protein tau".[19] To the surprise of some owners, their castrated dogs even show real sexual behaviour after years, which could also be due to the dopamine level, which can be stimulated by such behaviour.[20] The persistence of "male" behaviour after neutering is not due to potential remainders of sex androgens in the blood, since the testosterone in the plasma decreases within six hours after

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castration onto a low to no longer measurable concentration.[11] Furthermore, studies, in which animals were both castrated and adrenalectomised, excluded the possibility that the sexual behaviour of castrated animals is maintained by the adrenal androgens.[11]

It is a common theory that aggressive behaviour is generally related to testosterone and thus strives for ranking and competition for mating partners. However, for example, fear-related aggression is controlled by stress hormones such as cortisol and this in turn can be inhibited by testosterone. Thereby, a fear-enhancing effect and thus still unsafe behaviour can occur once the testosterone antagonist is no longer present after a castration.[20]

Some dog owners fear that owning a male dog directly amounts to undesirable behaviour and they are of the opinion to get around this by an early castration.[21] But in early castrated males it should be noted first of all that the testosterone is responsible for the healthy development of the penis and thus the penis and the penis bone can remain infantile and the foreskin can remain small and underdeveloped.[4]

These results are also supported by Beach et al,[22] who found that in males who have been castrated at birth, the penis never grew to the normal size. The closing of the growth plates of the bones is partly controlled by sex hormones. Animals that are neutered before this closing of the growth plates thereby experience prolonged bone growth. Furthermore, Zink et al[3] investigated the risk of developing cancer in the dog breed Vizsla and they found out that there is an increased risk for this disease in early castrated dogs. In a study of aggressive behaviour the finding was, that prepubertally castrated males are as aggressive as intact ones.[11] Also early castrated males seem likely to suffer more frequently from hip dysplasia (HD) than intact dogs, which could also be connected with the extended bone growth. Additionally, the risk of a cranial cruciate ligament rupture occurs twice as much in early castrated dogs.[4]

Little is known about the consequences of neutering on the social behaviour of dogs. In the first part of our study, the focus therefore lies on the social behaviour of male dogs among themselves. The question to be answered here is whether there are differences in certain behaviours between neutered and intact male dogs. There are several aspects of neutering that may found the assumption of behavioural differences. So the object of this paper is to contribute to a better understanding of the effects of neutering on the behaviour of male dogs by way of video recorded trials and two different questionnaires. Therefore, three hypotheses are formulated as follows: (1) Certainly shown in a different frequency by neutered and intact male dogs. (2) Neutered males show confident behaviour less frequently than intact males. (3) Intact and neutered males differ in their frequency of sending and receiving social behavioural responses.

In the second part two different questionnaires about a dog's behaviour and habits were distributed among owners of intact and neutered males.

In the second part, neutered and intact male dogs are being compared with respect to their trainability, boldness, calmness and dog sociability. Even though there is evidence for gonadectomy affecting a dog's cognitive abilities, a study on trainability in dogs found no clear correlation between neuter status and trainability.[19,23] Similarly, no difference in trainability of neutered and intact dogs is expected in this analysis. However, bearing in mind the findings of previous studies on excitability, fearfulness and aggression,[3,14,15,17,18] intact males are expected to be bolder, calmer and more sociable than neutered ones.

Finally, a more general comparison of neutered and intact male dogs is being conducted looking at various habits and behaviours in everyday life, such as restlessness, stereotypies and aggression in different situations; as well as at problems and difficulties owner are having with their dogs. In accordance with the previously mentioned hypothesis, intact dogs are again expected to be calmer and less aggressive than neutered ones. Also bearing in mind the findings of previous studies,[3,14] problems mentioned by owners of neutered males are predicted to include fearful behaviours, panic or hyperactivity more often than those mentioned by owners of intact dogs.

Overall, this study aims to contribute to a better understanding of the long-term effects of neutering on the behaviour of male dogs by analyzing the social behaviour of neutered and intact animals as well as by evaluating questionnaires submitted by dog owners.

EXPERIMENTAL METHODS

Video Analyses

Data on social behaviour was collected on groups of dogs consisting in equal parts of neutered and intact males in Germany and Switzerland (some of the dogs already knew each other from the dog schools). The dogs were released together into an enclosure and their behaviour was recorded using focal-animal-sampling with a video camera. Each individual was video recorded for 5 minutes. The first order of the focal animals was chosen randomly. In subsequent meetings, the order was rotated in a way that allowed for every dog to be recorded once in every position. This trial was conducted with six separate groups of dogs (17 intact, 16 neutered). The composition of the dog groups and further information about the participant dogs are shown in Table 1. For all groups, the meetings took place on the terrain of dog schools. In both cases, the dog owners were also inside the enclosures, as well as the owners of the dog school and one or two camera operators.

Questionnaires and Case Studies

The second phase of data collection consisted of the evaluation

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Table 1: Overview of the Participants of the Video Trials and their Classification to each Group.

Group No.	Label	Breed	Age	Neutering status
1	I1	Airedale Terrier	1 year	intact
	I2	German Shepherd	8 months	Intact
	N1	German Shepherd	5 years	neutered
	N2	Kangal mixed breed	9 years	neutered
2	I3	Grand Anglo Francois Tricolor	1 year	intact
	I4	Golden Retriever	1 year	intact
	N3	Labrador Retriever	3 years	neutered
	N4	Mixed breed	2.5 years	neutered
3	I5*	Barsoi	5 years	intact
	I5°	Belgian Shepherd	1 year	intact
	I6	Giant Schnauzer	3 years	intact
	I7*	English Springer Spaniel	1 year	intact
	I7°	English Mastiff	6 years	intact
	N5	Rhodesian Ridgeback	5 years	neutered
	N6	Kerry Blue Terrier	5 years	neutered
	N7	Australian Kelpie	2 years	neutered
4	I8	Labrador	4 years	intact
	I9	Chihuahua	2 years	intact
	I10	Shepherd-mix	4.5 years	intact
	I11	Boxer	3.5 years	intact
	N8	Newfoundlander	4 years	neutered
	N9	Schnauzer	10 years	neutered
	N10	Dachshund	4.5 years	neutered
	N11	Labrador-mastiff-mix	20 months	neutered
5	I12	Labrador retriever – Swiss – Briard - Mix	5 years	intact
	I13	Australian Shepherd	6 years	intact
	I14	Field Trial Labrador Retriever	3 years	intact
	I15	Papillon	-	intact
	I16	Bulldog	-	intact
	N12	Greater Swiss Mountain Dog	4 years	neutered
	N13	Leonberger	5 years	neutered
	N14	Labrador Retriever mix	7 years	neutered
	N15	Golden Retriever	-	neutered
	N16	Mix	-	neutered

'I' is used for intact males and 'N' is used for neutered males. A star (*) is used to label the males participating only in the first meeting of group 1, and the same label with a circle (°) for their respective replacements.

of a survey first established for a study in Budapest by Turcsán et al.[24] This questionnaire comprises a total of 24 questions about a dog's habits in various situations, which can be evaluated using a preset point system and therefore allows for providing individual scores on the four traits of trainability, boldness, calmness and dog sociability (Table 2). A total of 133 surveys completed by owners of male dogs were available for analysis, 29 of which were submitted by participants of the dog groups of the video analysis trials.

The questionnaires evaluated in the third part of the study were taken from archived files of dog owners taking part

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Table 2: Classification of the 16 Questions of the Questionnaires (based on Turcsán et al[24]) into the Four Personality Traits (Here it must be Emphasized that in Turcsan et al[24] "Boldness" is used as one of the 5 Feactors, Corresponding to the Factor Extraversion, not to the Supertrait "bold" of the Shy-Bold-Concept, e.g., in Behavioural Ecology.

Calmness	Trainability	Dog sociability	Boldness
My dog can be stressed easily	My dog is very easy to warm up to a new toy	My dog fights with conspecifics frequently	My dog is unassertive, aloof when unfamiliar persons enter the home
My dog is emotionally balanced and not easy to rile	My dog is ingenious, inventive when seeks hidden food or toy	My dog is enthusiastic and animates (encourages?) other dogs to play	My dog is rather cool, reserved
My dog is cool-headed even in stressful situations	My dog often does not understand what was expected from him/her during playing	My dog is suspicious (mistrustful? leery?) of other dogs	My dog is sometimes fearful, awkward
My dog is sometimes anxious and uncertain	My dog is not much interested except in eating and sleeping	My dog is often bullying with conspecifics	
	My dog is intelligent, learns quickly		

in an animal behaviour consultancy program called Einzelfelle (www.Einzelfelle.de) by zoologist PD Dr. Udo Gansloßer and veterinarian Sophie Strodtbeck. In the course of this program, people seeking advice in the management of their pets were asked to give their reasons for seeking council and to fill in questionnaires consisting of questions about their dog's environment, health, habits and obedience. The files of 54 participants (27 intact and 27 neutered males) were examined regarding the reasons for participation in the program. They included fully completed questionnaires which were evaluated as to various behaviour patterns in everyday situations (Table 3). None of the neutered dogs referred to consultancy were castrated for reasons of aggression, stress or similar specific "diagnosis". Instead there were dogs castrated because it was thought customary to do so or for reasons such as keeping with an intact bitch, being prevented to enter dog- daycare facilities otherwise, routinely in animal shelters, etc.

Data Analyses

The analysis of the video recordings of social behaviour was conducted using an ethogram originally created by Goodmann et al[25] and modified by Spitzley and Elsing as well as Feddersen-Petersen (for detailed description of included behaviours see Appendix, Ethogram).[26] All included behaviours were recorded as either sent by or received by the focal individual. For behaviour patterns seen as events, the number of occurrences was counted. For state behaviours the total duration of the behaviour was recorded to the nearest second.

Statistical and graphical analyses of the behaviour recordings were performed using the statistical program SPSS.[24] Significances of frequencies were calculated using a Mann-Whitney-U-test for two- tailed independent data and a Kruskal-Wallis-Test for dependent data. Additionally a one-

Table 3: List of Issues Mentioned as Reasons for Seeking Council in Dog Management as well as of Behaviour Patterns Examined in this Paper. The Information of both Reasons and Behaviour Patterns was Obtained from Questionnaires using yes-or-no Questions. The Owners were Asked to fill in these Parts by Ticking for "yes".

Reasons for seeking council	Behaviour patterns
Consideration of neutering	Restlessness
Epilepsy	Dog is never tired
Thyroid abnormalities	Nervousness
Stress/insecurity	Absent-mindedness
Panic	Shaking
Hyperactivity	Panting without heat
Aggression	Excessive licking/scratching
Else	Stereotypic behaviour
Barking/whining	
House-training unsuccessful	
Lead pulling	
Excessive demand for attention	
Aggression against dogs	
Aggression against humans	
Chasing	

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way ANOVA (randomization test) of the behavioural states was conducted *via* the online statistical program "StatKey" (http://www.lock5stat.com/StatKey/). The *p*-values were calculated by an online calculator (http://www.socscistatistics.com/pvalues/fdistribution.aspx). However, not all 33 dogs, but only the recordings of 26 dogs could be analyzed in the ANOVA.

The results of the BUDAPEST surveys and the Einzelfelle questionnaires were evaluated descriptively and depicted as graphs using SPSS Version 24 as well as Microsoft Excel (2010).

RESULTS

Video Analyses

For the evaluation of social behaviour amongst neutered and intact male dogs, an ethogram consisting of 23 behavioural patterns was used. The analysis was conducted in two parts, the first of which was an evaluation of behaviour sent by focal dogs whereas the second part considered received behavioural patterns. There are significant differences among the behavioural patterns 'molest', 'Sniffing of anal region', 'Sniff genitals', 'Pose' and 'Chinrest' between the intact and castrated dogs. All these behaviours were recorded from more intact than neutered male dogs. Moreover the 'Toothchatter' represents a behavioural pattern only shown by intact ones (Figure 1).

For the confident=assertive behaviours, which were a sample category of our ethogram, consisting of the following elements 'chinrest', 'T-formation' and 'overmark', the plotted data show that there are big differences for the behaviour of 'chin rest' between the neutered and intact male dogs. Equally the frequency of sending 'T-Formation' is higher by the intact dogs. The same applies to 'Overmarking', which also occurred more frequently in intact than castrated dogs (Figure 2).

Figure 3 shows that the castrated males have a significantly lower proportion of sending social behaviour (19%) than the intact dogs (33%). Whereas in terms of receiving social behaviours, the percentage of the neutered dogs (27%) is higher than of the intact males (21%).

Certain behavioural patterns like 'Sniffing of anal region', 'Nosing' and 'Sniffing the inguinal region', are shown more frequently by intact than neutered males with the exception, that the median is the same for 'Sniffing inguinal region' (Figure 4). Keeping the 'Ears pricked sideways' can be recorded more

Figure 1: Percentage of the Frequencies of each Behavioural Pattern sent by Neutered and Intact Dogs (n=658). ANALREG=Sniffing of Anal Region, SNIFFGEN = Sniff Genitals. The Statistical *p*-Values (Mann-Whitney-U-Test) are as Following: Molest: *p*=0.001; Analreg: *p*=0.005; Toothchatter: *p*=0.006; Sniffgen: *p*=0.020; Pose: *p*=0.015; Chinrest: *p*=0.061; Earsside: *p*=0.342; Huddle: *p*=0.488.

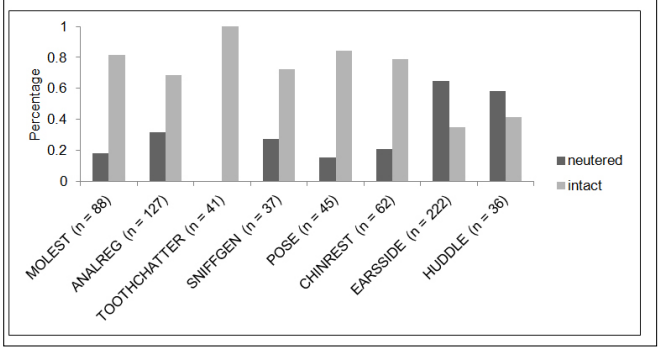


Figure 2: Comparison of Frequencies of the Three Confident Behaviours 'chin rest', 'T-formation'(TF) and 'Over Mark' sent by Intact and Neutered Males (n=260).

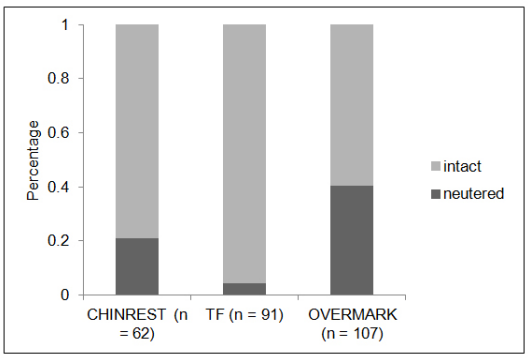


Figure 3: Percentage of the Frequency of all Behavioural Patterns Sent and Received by Neutered and Intact Dogs (SenderIntact: n=905; Sender Neutered: n=525; Receiver Intact: n=562; Receiver Neutered: n=738; Total n=2730). The stastical *p*-values (Mann-Whitney-U-Test) are as Following: Sender Intact/Sender Neutered: *p*<0.0005; Receiver Intact/ReceiverNeutered: *p*=0.280; Receiver Intact/Sender Intact: *p*=0.026; Receiver Neutered/Sender Neutered: *p*=0.184.

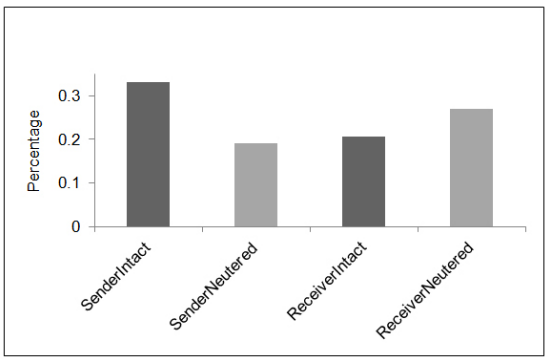
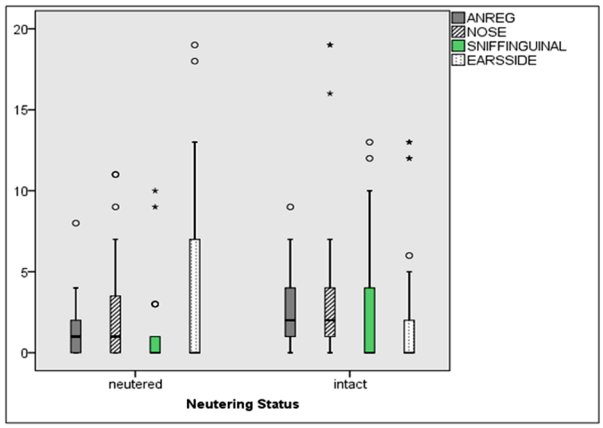


Figure 4: Comparison of the Frequencies of Each Behavioural Pattern Sent by Neutered and Intact Dogs (n=34 Dogs). ANREG=Sniffing of Anal Region, NOSE=Nosing, SNIFFINGUINAL=Sniff Inguinal region, EARSSIDE=Ears pricked sideways. The statistical *p*-values (Mann-Whitney-U-Test) are as following: Anreg: *p*=0.005; Nose: *p*=0.077; Sniffinguinal: *p*=0.158; Earsside: *p*=0.342.



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for castrates, but here again there is no difference between the median values.

Statistical Analyses

The statistical analysis of the social behaviours frequently sent by the neutered and intact dogs reveals significant differences between the castrates and the intact dogs (Figure 5) (see Appendix, Table 4). Highly significant results can be found in the behavioural patterns of 'Molest' (Mann-Whitney-U-Test, U=382.000, p=0.001), 'Sniffing of anal region' (Mann-Whitney-U-Test, U=568.000, p=0.005), 'Licking genitals' (Mann-Whitney-U-Test, U=475.000, p=0.008) and 'Toothchattering' (Mann- Whitney-U-Test, U=575.000, p=0.006). The behaviours 'Sniffing genitals' (Mann-Whitney-U-Test, U=464.000, p=0.020), 'Pose' (Mann-Whitney-U-Test, U=492.500, p=0.015) and 'T-formation' (Mann-Whitney-U-Test, U=475.500, p=0.018) differ significantly. At last the two behaviours 'Nosing' (Mann-Whitney-U-Test, U=465.000, p=0.077) and 'Chinrest' (Mann-Whitney-U-Test, U=492.500, p=0.061) reflect a trend among the castrated and non-castrated dogs. When looking at the statistical analysis of the behavioural patterns as 'states' (see Appendix, Table 5), significant differences or at least trends can be found for the same behaviours as those as shown in the analysis of the frequencies (see Appendix Table 6). But apart from these nine behavioural events (molest, sniffing the anal region, licking genitals, tootchchatter, sniff genitals, posing, T-Formation, chinrest and nosing), two other behavioural patterns are statistically relevant, if the video trials are analysed for their duration. These behaviours are 'Sniffing the inguinal region' (F=6.203; p=0.016) and 'Inguinal presentation' (F=2.714; p=0.105).

Questionnaires

The results of the BUDAPEST Questionnaires for the 67 intact and 66 neutered males reveal that the intact males show a higher median for boldness (Here it must be emphasized that in Turcsan et al[24] "boldness" is used as one of the 5 factors, corresponding to the factor extraversion, not to the supertrait "bold" of the shy-bold-concept, e.g. in behavioural ecology.) (median=5) than the castrates (median=4) (Figure 6). For the category 'Sociability to dogs' the scores of the neutered dogs are also lower (median=5) than the values of the intact males (median=6). Regarding calmness no difference can be noticed between the median values of both the neutered and intact dogs (median=3). The situation is different with the feature trainability. Here, the neutered males have a higher median value (median=9) than the non-castrated dogs (median=8).

Case Studies

A percentual comparison of intact and castrated males regarding behaviours selected from the anamnesis questionnaires is shown in Figure 7. The biggest difference between castrates and intact males can be found for fearful behaviour. Here, 15 of the 17 castrated dogs show anxiety during the walk. By contrast, this has been noted only for five intact dogs. Aggression towards other dogs can also be counted for more castrated and less intact dogs. Twelve intact males show fear of situations and among the castrates 14 exhibit such a behaviour. Hunting behaviour occurs to the same extent in the castrated and intact males.

The reasons of dog owners giving rise to contact Udo Ganslosser and Sophie Strodtbeck are shown in Figure 8.

Concerning multi-dog household, there are more owners of castrated dogs than owners of intact males, who decided to get a behavioural medical recommendation. Epilepsy can also be noticed for more intact than neutered males and the thyroid gland represents the reason for three castrated and for twice as many intact dogs. Stress or insecurity is indicated for both ten

Figure 5: Comparison of the Duration of each Behavioural Pattern Sent by Neutered and Intact Dogs (n=34 dogs). The Statistical p-Values (by an ANOVA Randomization Test) are as Following: Sniffing of Anal Region: p=0.011; Nosing: p=0.056; T-Formation: p=0.007; Shelter: p=0.110; Sniff Inguinal Region: p=0.016.

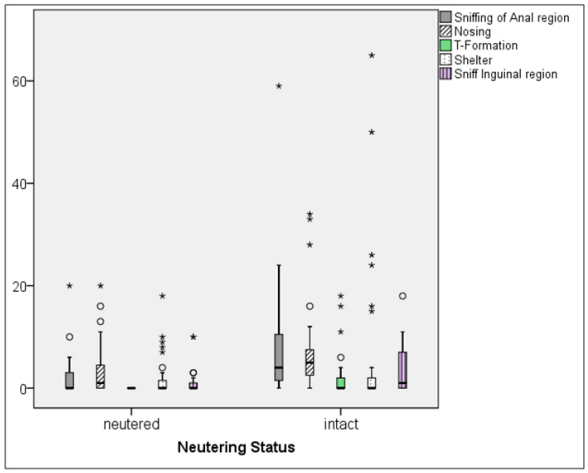

Figure 6: Evaluation of the BUDAPEST Questionnaires Regarding 67 Intact and 66 Neutered Dogs (n=133 dogs).

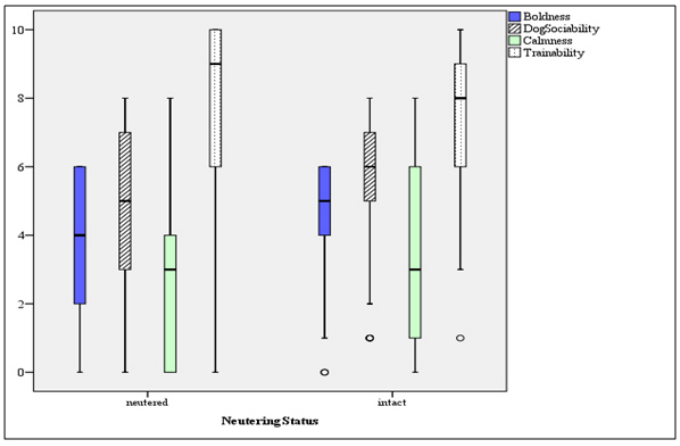

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Figure 7: The Percentage of Selected Behaviours of 34 Questionnaires of the 54 Case Studies (n=94).

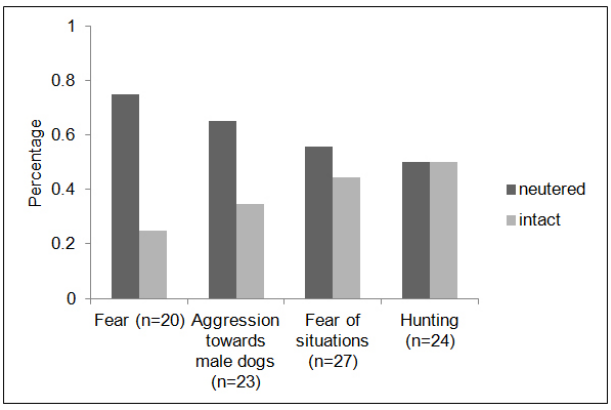


Figure 8: Comparison of Reasons for Seeking Council Given by Owners of Neutered and Intact Male Dogs (n=133 dogs).

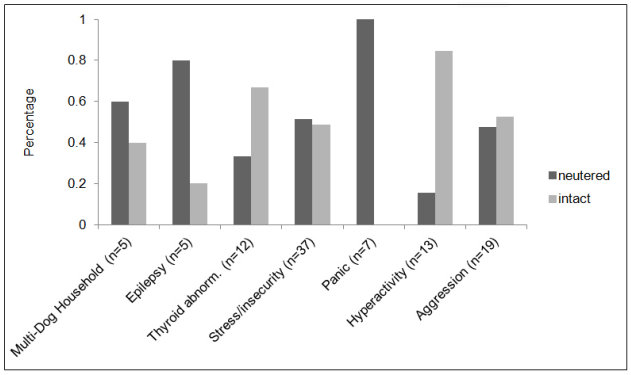
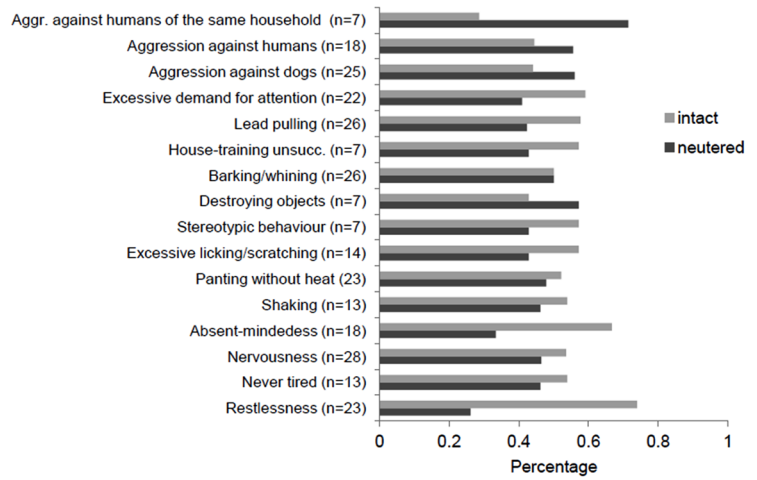


Figure 9: Percentage of Neutered *versus* Intact Males Affected by Several Investigated Behaviours (n=17 dogs).



neutered and intact dogs. Panic can be noticed only for neutered and for none of intact males. Hyperactivity is shown significantly more by intact than neutered dogs. Finally, aggression can be recorded more frequently in the castrated males.

A graphical analysis of 54 completed Einzelfelle questionnaires revealed that of 16 considered behaviours, 11 were shown more often by intact males (Figure 9). However, barking and/or whining can be noted equally in neutered and intact dogs, and neutered males also tend to be more aggressive towards both humans and humans of the same household as well as towards dogs. Destroying objects is also noticed for more castrates than intact ones.

DISCUSSION

Video Analyses

The aim of this study was to gain further insight into the effects of neutering on a male dog's behaviour by comparing the social behaviour of neutered and intact animals in an observational part as well as by evaluating questionnaires submitted by dog owners.

We found significant differences among several behavioural traits (e.g., molest, sniffing the anal region, licking genitals, tootchchatter, sniff genitals, posing, T-Formation, chinrest and nosing), verifying our first hypothesis, which implies that there are certain behaviours which are shown in a different frequency by neutered and intact male dogs. In particular, these significant differences show that the neutered dogs are more likely to receive 'sniffing of the anal region' from intact males. Dogs, like many other mammals, use pheromones and other scents for social differentiation. The sniffing of the "anogenital" region and of the head as well as the close following of a conspecific are summarized as an olfactory inspection.[27] In canids, three functions of anal gland secretion are distinguished: on the one hand, the sexual attractiveness is taken into consideration and on the other hand, it is associated with individual recognition and territorial delineation. They are

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also assigned an alarm function.[28]

The fact that castrated male dogs are more frequently harassed and sniffed, and that their genital and inguinal area are more frequently examined by the intact males, indicates a possibly increased attractiveness of the castrated males in relation to the intact males. Preferably, the genital region of bitches or faeces is intensively inspected and also licked in the estrus phase. Usually, animals in a relaxed mode allow each other to smell this region.[25] In our video recordings, however, the castrated male dogs did not appear to feel comfortable and the resulting stress can be illustrated by the lateral placement of the ears of the castrates, which also represents a significant difference. According to the ethogram, the 'ears pricked sideways' represents an advertising behaviour on the one hand, but it also occurs when a dog is stressed.

The tooth chattering is probably for the most part in a sexual context, since it occurs within the video recordings predominantly during the sniffing of the genital or inguinal region. This behavioural pattern is also described as nibbling from the male, which examines the female genital area. Thereby the incisors are quickly thrown together.[25]

In the case of confident/assertive behaviour it can be shown that the second hypothesis ('Neutered males show confident behaviour less frequently than intact males') is also verified regarding the behaviour of chinrest and T-formation. Despite the fact that some of the neutered dogs are older than the intact dogs, the latter appear more sovereign in relation to the neutered ones.

The T-formation is often used by males who approach bitches in their heat. In this case of our study, the intact males approach the neutered dogs more frequently, which again gives an indication for the increased attractiveness of the castrated males concerning the intact dogs. This formation is also related to assertive behaviour, in which case the self-confident animal occupies the crossbar position.[25]

The behaviour pattern 'chin rest', as found e.g., by Dopfer[29] in foxhounds, does not necessarily have a connection to dominance as a social relationship. This confirms our data that it can also occur in the context of assertive behaviour and is also an approach. On the basis of the video recordings, more intact than castrated males rested their chin on the neutered dogs, and consequently appear more confident in their behaviour than the neutered dogs. Testosterone is associated with anxiety, and men with low testosterone levels are more likely to suffer from anxiety and depression, which may be decreased by a treatment with testosterone.[21] An important aspect probably explaining the uncertain behaviour of the neutered dogs is the lack of testosterone.

The differences between sending and receiving of social behaviour coincide with the above-mentioned results, because here again the intact dogs are sending social behaviour more frequently than the castrated males. When a specifiatc signal is sent, it leads to a specific response by the receiver, as long as it has been received correctly. Methodically, however, there is the problem that not every communication taking place between animals is automatically recognized as such by human. It can sometimes happen that a signal is not understood by the receiver or deliberately ignored and thus it is overlooked as a real signal, since the reaction is not visible or does not occur as expected.[30]

At overmarking, even the castrates show this behavioural pattern more frequently than the intact dogs but no significant difference can be made in this regard. This behavioural complex is dependent on testosterone, in which the urine of an intact bitch is over-marked by the intact male. However, it is important to distinguish whether a male "directly hits the brand of his or her forerunner or beneath" and the latter is independent of testosterone.[31]

Testosterone probably does have an important inpact on the urine composition. In a study by Raymer et al[28] on the chemical composition of the urine in canids, it was found that certain substances were not present in the urine of the castrates. By treatment with testosterone, however, the neutered males had the same concentration of these substances as the intact males. But the responsible mechanism of the hormones could not be clarified in the study so far. In another study, the urine composition of the wolf and its results indicate that testosterone administration in castrated males causes the formation of certain components associated with intact males, while at the same time decreasing the concentration of other substances that are associated with neutered ones and females. This again emphasizes the hormonal influence on certain chemical fragrance components, which are important for the communication. In addition, the observation that almost two thirds of the investigated behaviour patterns were observed longer or more frequently by intact males might point towards them being more socially active in general. An increase in sample size might offer more information in that field. Furthermore, the fact that there are no significant differences in those behavioural patterns (like mounting, overmarking, urinating with raised leg) between the neutered and intact dogs, that are the most common reasons for neutering, shows that there is no change or improvement in these behaviours.

Questionnaires

Looking at the results of the questionnaires, it can be noted, that the neutered dogs appear less socially to other dogs and also less calm. In the behavioural category 'Boldness', the higher score of the intact males indicates that the intact dogs also react more relaxed and emotionally stable in stressed situations than the castrated dogs (Here it must be emphasized that in Turcsan et al[24] "boldness" is used as one of the 5 factors, corresponding to the factor extraversion, not to the supertrait "bold" of the shy-

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bold-concept, e.g., in behavioural ecology.). The intact dogs reach a higher score in the category 'Dog sociability' and also a higher median, whereby giving a lower tendency for mistrust and fighting than the castrated males. In contrast, the neutered dogs seem to have a higher tendency for bullying and aggressive conflicts. At 'Trainability', the overall score of the neutered dogs is higher than that of the castrated males, so there seems to be an improvement in training a dog, when it is neutered. Nevertheless, all these results are based on the observations of dog owners and cannot be regarded as completely neutral and objective observations.

Case Studies

The results of the case studies regarding to specific behaviours indicate that the castrated male dogs show more anxiety on walks than the intact ones. Likewise, there are more neutered than intact males showing aggressive behaviour against other males. In addition, more castrated dogs show fear of situations. In an article of Farhoody and Zink, published in 2010,[32] which is based on a master thesis, the results from the surveys are summarized with a sample size of more than 10,000 dogs. This study indicates that dogs develop a more anxious behaviour after neutering, because they are denied their ability to explore their surroundings and to process the experiences of fear in a correct manner. Among other things, it was also pointed out in our project that castrated dogs were more aggressive, more anxious and more excited than the intact dogs. The data from the study mentioned above showed significant correlations between a castration and increased aggression, anxiety and restlessness, regardless of the age at which the castration was performed.[31]

Nevertheless, there are studies being contrary to our results. These are related to a decrease or disappearance of behavioural problems in 74% of male dogs after neutering[33] or to behavioural improvement on aggression toward humans is approximately 25% of adult dogs after gonadectomy.[34] Some discrepancies between our findings and those of e.g., Zink et al[3] on the one hand, and data by Heidenberger and Unshelm[33] and Neilson et al[34] on the other hand, who found that some forms of aggression might be reduced by neutering, may be explainable by the fact that aggression is not a unique behavioural system with one common underlying motivation, physiological causation or evolutionary function but a reaction style that can be caused by very different underlying motivations, previous experiences or stimuli.[37]

With regard to the increasing rate of castration with the aim of changing undesired behaviour, some facts should be kept in mind: firstly, which behaviours are impaired and then: What is the probability that a specific behaviour is really influenced. On the other hand, the dog's experiences before a castration also play a role in maintaining a behavioural pattern after neutering. Furthermore, the differences between the different breeds should also be considered.[35]

Also striking is the difference in hyperactivity between the neutered and intact dogs. Here it is indicated for more intact than castrated males. The reason for this could be that the castrates might appear unenthusiastic or are generally less active. Niepel[5] describes in her book that some owners (5%) stated that their dog was less active and some (13%) said he had become more lethargic. She is of the opinion that *via* neutering there is a tendency for changing the dog's activity, but not being clear in which direction. Thus, a male is not automatically quieter by a castration.[5]

Regarding aggressive behaviour, it is noticeable that the castrates are more likely to exhibit some of this behaviour than the intact males, which in turn supports the observation that not all types of aggression can be influenced positively by a castration.[5] In more neutered than intact dogs, other reasons are listed, and some of them, such as aggression against people, in the same household, stereotypes, nervousness, panic at loud noises and sensitivity towards emotions (meaning emotional instability) again indicate the behavioural consequences of a castration, also supported by Zink et al[3] in castrated Vizslas. Regarding stress hormones again, as already described in the introduction, it becomes clear that fear-related aggression is controlled by cortisol and testosterone is eliminated as an inhibitor by a castration.[36] Finally, aggressive behaviour is a multi-functional category, thus as a diagnosis it would have to be much more specified. Archer[37] distinguishes at least three main categories of aggression, i.e., self-protective, parental and competitive, and only some parts of the latter category are influenced by gonadal hormones.

All these results suggest the aspect of the placebo effect in which the owners after a castration could have expected a change in a specific behaviour in some way, and possibly estimate their neutered dogs as positively changed in this behavioural pattern.[35] Also, at the end of adolescence the undesirable behaviour might decrease anyway, regardless of previous castrations.

Various problems may occur in such a study. During the recording sessions, it was not possible to have all dogs present on all occasions. It cannot be excluded that the group composition affects individual behaviour in such a trial. Observed differences in behaviour might therefore also be connected with the absence of a group member of the difference in neutered/intact ratio. Also, dog owners were present during the recordings, which might have affected their dogs' behaviour. Indeed, some owners intervened during the trials when their dog showed behaviour they judged as overly aggressive or undesirable.

CONCLUSION

The results do not automatically lead to the conclusion that all neutered dogs are more frequently molested than intact males, but a clear tendency is evident that neutered dogs differed in their social behaviour from intact males. One important point could

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also be the mean age differences between the dog groups, which can mean a limitation of interpretation of social behavioural differences. The results may perhaps be different if the dogs were of the same age.

The results of the Budapest questionnaires and also those of the case studies confirm this thesis. Neutered dogs are more likely to become more anxious and insecure, which is already supported by other research.[3] This aspect, as well as animal welfare, should never be disregarded before deciding for a castration.[5]

The knowledge to be gained is very important for present and future dog owners as well as veterinarians with particular reference to the fact that neutering of dogs has become part of 'responsible ownership' in many countries, which according to our results might not be so responsible at all, at least not in all cases.

Understanding long-term effects of the procedure on dog behaviour will be essential for deciding whether or not to neuter a dog, especially since it is often done not to prevent breeding but precisely to change behavioural aspects of a pet.[5,16] Even now we could go so far as to advise dog owners against neutering with the intent to correct undesired habits because the lack of verifiable difference in and of itself is enough to question the justifiability of such a drastic intervention, at least until more information can be provided on the behavioural changes induced by gonadectomy. This paper may serve as a forerunner to follow-up studies looking at specific aspects of social behaviour in intact and neutered dogs.

In order to gain a broader view into the subject of castration and its consequences with regard to social behaviour, a comparison with an analysis of bitches would be desirable and so we are in a process of conducting a research on neutered bitches with a parallel study design, which will be published in the future.

In the end, both males and females are always dependent on the decision of the owners, and these should not decide imprudently. The health and well-being of the dog should always be a priority. Therefore, veterinarians should also provide the dog owners all possible alternatives to a castration in order to get the most appropriate decision for the dog.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest whatsoever.

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APPENDIX

Ethogram

SNIFFING OF ANAL REGION (ANREG)

Sniffing of the anal region of another individual. This is thought to serve the establishment of social contact between dogs.

HUDDLE

Seeking of close physical contact to a conspecific or a human by sliding and rubbing up against the social partner's body.

RIDE UP

Often standing in a right angle to another dog, the front paws are laid onto or over its back.

MOLEST

Repeated and unrequited seeking of contact to a conspecific, threatening and aggressive behaviour. The molesting dog tries to prevent the other one escaping.

NOSING (NOSE)

Slow and intense nosing of another dog, sometimes circular movements of the nose along the other dog's body.

MOUNTING (MOUNT)

Grasping another dog between the front legs, normally from behind but sometimes from the front.

FOLLOW (FOL)

Following another dog and changing directions with him, sometimes keeping a constant distance.

SNIFF GENITALS (SNIFFGEN)

Sniffing of the genital region and around the genitals, often seen during first contact. Can be avoided by the dog being sniffed by tucking in his tail and bending his back legs.

LICK GENITALS (LICKGEN)

Licking of genitals, sometimes also done to puppies to stimulate digestion or to females in heat.

SNIFF INGUINAL REGION (SNIFFING)

Sniffing of the area of the inner thighs as well as of the belly between and behind the knees.

INGUINAL PRESENTATION (INGPRES)

Lifting of one back leg to facilitate the sniffing of the area of the inner thigh.

CHIN REST

Placing of the head on another dog's back or shoulders, sometimes observed as confident behaviour, but can also be used in a socio-positive context as well as in preparation of mounting.

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APPENDIX

EARS PRICKED SIDEWAYS (EARSSIDE)

Ears are lifted upright and turned in a way that the inner part is pointed to the sides, sometimes to a point where the backs of the ears are almost touching.

POSE

The dog is standing upright with bent neck and his ears typically pricked upright or turned sideways.

TAIL AVERT (TAILAV)

The tail is bet sideways at the root to hang alongside the hip. This is a signal of females ready for mating.

T-FORMATION (T-FORM)

One dog places himself at right angles to another, which looks like the letter 'T' from above.

OVER MARK (O-MARK)

One dog urinates over the urine marking of another.

URINATE

Releasing of urine.

RAISED LEG URINATION (RAISEDLEG)

Urinating by lifting one leg and holding it at right angles to the body.

TOOTH CHATTER (TOOTHCH)

Incisors are brought together several times in rapid succession to bring olfactory substances to the vomeronasal organ. This behaviour is often combined with excessive salvation.

SHELTER

The dog draws back and finds shelter, sometimes if he is the goal of molesting or an attack, but also if a conspecific is attacked.

SHAKE

The whole body is shaken, often starting from the snout, along the longitudinal axis.

YAWN

Wide opening of the jaw combined with taking a deep breath. The eyes are often pressed closed and the tongue rolled upwards.

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APPENDIX

Table 4: Behavioural Patterns Sent by Neutered and Intact Dogs (n=33) as Frequencies in Total and as Percentage Performed by Neutered *Versus* Intact Males. Significance was Tested using a Two-Sided Mann-Whitney-U-test test.

	Total		Percentage		Median		*p*-Value
	Neutered	Intact	Neutered	Intact	Neutered	Intact	(Mann-Whitney U Test)
MOLEST	16	72	18%	82%	0	0	0.001 ***
ANALREG	40	87	32%	68%	1	2	0.005 ***
LICKGEN	7	41	15%	85%	0	0	0.008 ***
TOOTHCHATTER	0	41	0%	100%	0	0	0.006 ***
SNIFFGEN	34	81	29%	71%	0	0	0.02**
POSE	7	38	16%	84%	0	0	0.015 **
TF	4	87	4%	96%	0	0	0.018 **
CHINREST	13	49	21%	79%	0	0	0.061 *
NOSE	82	122	40%	60%	1	2	0.077 *

*= trend (p ≤ 0.1); **=significance (*p*≤0.05); ***=highly significant (*p*≤0.01). TF=T-Formation; ANALREG=Sniffing of anal region; SNIFFGEN=Sniff genitals; LICKGEN=Lick genitals.

Table 5: Behavioural Patterns Sent by Neutered and Intact Dogs (n=26) as States in Total and as Percentage Performed by Neutered *Versus* Intact Males. Significance was Tested using a one-way ANOVA Randomization Test for Differences in Means.

	Total		Percentage		Median		*p*-Value
	Neutered	Intact	Neutered	Intact	Neutered	Intact	Randomization Test
MOLEST	9	42	18%	82%	0	1	0.001***
POSE	0	7	0%	100%	0	0	0.004***
TF	0	23	0%	100%	0	0	0.007***
ANALREG	71	119	37%	63%	0	4	0.011***
SNIFFINGUINAL	33	36	48%	52%	0	1	0.016**
SNIFFGEN	5	19	21%	79%	0	0	0.028**
LICKGEN	0	12	0%	100%	0	0	0.034**
TOOTHCHATTER	0	16	0%	100%	0	0	0.049**
NOSE	115	142	45%	55%	1	5	0.056*
CHINREST	5	8	38%	62%	0	0	0.656*
INGPRES	0	16	0%	100%	0	0	0.105*
SHELTER	115	142	45 %	55 %	**1**	**5**	0.110
EARSSIDE	5	8	38 %	62 %	**0**	**0**	0.116

*=trend (*p*≤0.1); **=significant (*p*≤0.05); ***=highly significant (*p*≤0.01). TF=T-Formation; ANALREG=Sniffing of anal region; SNIFFINGUINAL=Sniff inguinal region; SNIFFGEN=Sniff genitals; LICKGEN=Lick genitals; NOSE=Nosing; INGPRES=Inguinal presentation; EARSSIDE=Ears pricked sideways.

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APPENDIX

Table 6: Statistical Results of the Randomization Test of the Behavioural Patterns Sent by Neutered and Intact Dogs (n = 26) as States in Total Performed by Neutered *Versus* Intact Males. Significance was Tested using a One-Way ANOVA Randomization Test for Differences in Means.

	Mean neutered	Mean intact	Mean overall	Standard Deviation neutered	Standard Deviation intact	Standard Deviation overall	dfn	dfd	F-ratio value	*p*-value	Sign.
MOLEST	0.3	5.7	3.0	1.3	8.2	6.4	1	60	13.044	0.001	***
POSE	0.0	3.1	1.5	0.0	5.6	4.2	1	60	9.175	0.004	***
T-FORM	0.0	2.3	1.1	0.0	4.6	3.4	1	60	7.763	0.007	***
ANALREG	2.3	7.9	5.1	11.7	4.6	8.8	1	60	6.818	0.011	***
SNIFFING	1.1	3.5	2.3	2.5	4.8	4.0	1	60	6.203	0.016	**
SNIFFGEN	0.2	1.1	0.6	0.6	2.3	1.7	1	60	5.073	0.028	**
LICKGEN	0.0	5.1	2.5	0.0	13.0	9.5	1	60	4.734	0.034	**
TOOTHCH	0.0	1.0	0.5	0.0	2.7	1.9	1	60	4.014	0.050	**
NOSING	3.7	7.4	5.5	5.4	8.9	7.5	1	60	3.791	0.056	*
CHINREST	0.2	1.8	1.0	0.5	4.8	3.5	1	60	3.518	0.066	*
INGPRES	0.0	0.2	0.1	0.0	0.7	0.5	1	60	2.714	0.105	*
SHELTER	2.0	6.6	4.3	4.2	15.4	11.4	1	60	2.509	0.110	
EARSSIDE	56.8	30.1	41.4	77.6	51.8	66.8	1	60	2.546	0.116	
OVERMARK	0.4	1.4	0.9	1.2	4.6	3.4	1	60	1.261	0.266	
RAISEDLEG	0.6	1.2	0.9	1.4	3.0	2.3	1	60	1.096	0.299	
HUDDLE	0.5	1.5	1.0	1.7	6.5	4.7	1	60	0.780	0.381	
FOLLOW	27.4	39.9	34.0	27.4	39.9	34.0	1	60	0.371	0.545	
URINATE	0.9	0.7	0.8	1.6	1.5	1.5	1	60	0.326	0.570	
MOUNT	0.6	0.7	0.7	2.2	1.9	2.1	1	60	0.034	0.854	
SHAKE	0.5	0.5	0.5	0.8	0.7	0.7	1	60	0.029	0.865	
RIDE UP	0.8	0.8	0.8	2.4	3.1	2.7	1	60	0.0021	0.964	
TAILAV	0.0	0.0	0.0	0.0	0.0	0.0	1	60	-	1.0	
YAWN	0.1	0.1	0.1	0.4	0.2	0.3	1	60	0.000	1.0	

It is indicated by Dark blue ***=highly significant ($p \leq 0.01$); blue **= significant ($p \leq 0.05$); light blue *=trend ($p \leq 0.1$). T-FORM=T-Formation; ANALREG=Sniffing of anal region; SNIFFING=Sniff inguinal region; LICKGEN=Lick genitals; TOOTHCH=Tooth chatter; INGPRES=Inguinal presentation; EARSSIDE=Ears pricked sideways; RAISEDLEG=Raised leg urination; TAILAV=Tail avert. Dfn=degrees of freedom numerator; dfd=degrees of freedom denominator. Sign.=Significance