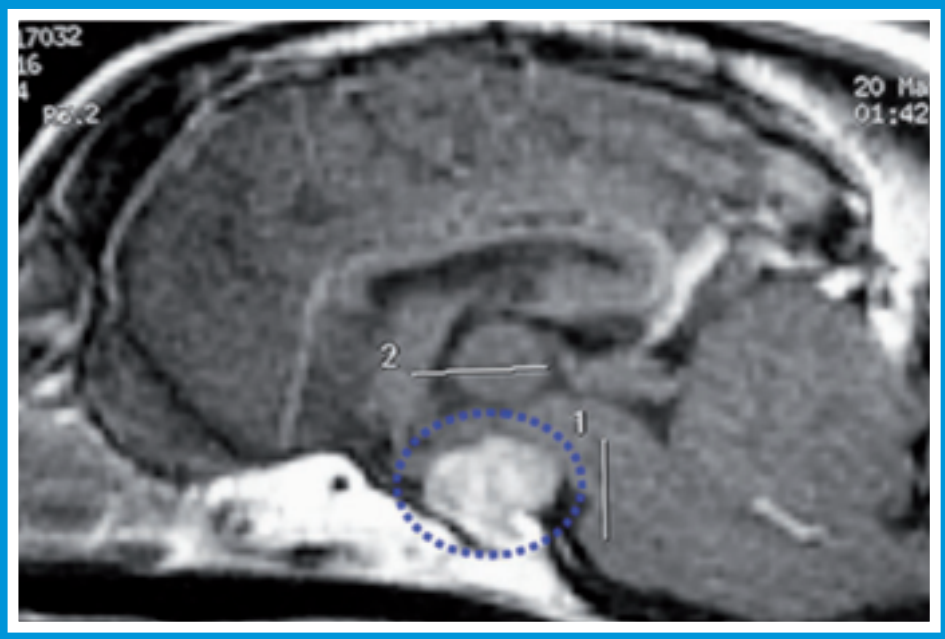
ISSN 1580-4003

THE SCIENTIFIC JOURNAL OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA

# SLOVENIAN VETERINARY RESEARCH

## SLOVENSKI VETERINARSKI ZBORNIK




Slov Vet Res 2009; 46 (3): 81-120

Volume 46 3

Slov Vet Res • Ljubljana • 2009 • Volume 46 • Number 3 • 81-120

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The Scientific Journal of the Veterinary Faculty University of Ljubljana

**SLOVENIAN VETERINARY RESEARCH**
**SLOVENSKI VETERINARSKI ZBORNIK**

Previously: RESEARCH REPORTS OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA
Prej: ZBORNIK VETERINARSKE FAKULTETE UNIVERZA V LJUBLJANI

4 issues per year / izhaja štirikrat letno



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Sponsored by the Slovenian Research Agency
Sofinancira: Agencija za raziskovalno dejavnost Republike Slovenije

ISSN 1580-4003
Printed by / tisk: Birografika Bori d.o.o., Ljubljana
Indexed in / indeksirano v: Agris, Biomedicina Slovenica, CAB Abstracts, IVSI
Urlich's International Periodicals Directory, Science Citation Index Expanded,
Journal Citation Reports/Science Edition
http://www.slovetres.si/

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**Slov Vet Res 2009; 46 (3)**

Slov Vet Res 2009; 46 (3): 85-91
UDC 57.085:577.175.6:612.6.052:612.823

**Review Paper**

# IS MALE BRAIN DIFFERENT FROM FEMALE BRAIN?

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**Summary:** In 1959, exactly 50 years ago, was published a paper by Phoenix, Goy, Gerall and Young entitled "Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig". Before the publication of this paper, it was widely accepted that hormones do act upon brain. However, the general thought was that hormones, especially sex steroid hormones, directly activate certain brain areas when needed, i.e. at the time of mating, parental care etc. In contrast to this thought, Phoenix and colleagues for the very first time proposed that hormone action in neonatal period could also permanently alter brain structure, and thus influence differences in behavior long after exposure to sex steroid hormones. The study of Phoenix and colleagues was therefore revolutionary, and as such, had many opponents at that time. Even the authors themselves were very cautious in their phrasing, never directly claiming that hormones could alter brain structure but rather even in the title used the words "tissues mediating mating behavior" instead of brain or central nervous system. Furthermore, as with many such revolutionary studies, study by Phoenix and colleagues left more questions unanswered than it did answer. The authors did not and could not know at that time exactly where and how do steroid hormones act in the brain, they did not know whether observed effects in their study arose from the direct action of testosterone or perhaps from some testosterone metabolite. In half the century since the publication of this seminal study, hundreds of papers have been published, confirming initial finding of Phoenix and colleagues, and these papers have provided answers to many questions raised by the authors. Today we know that at least in rodents, it is testosterone metabolite estradiol that masculinizes the brain. We know that brain structure could be altered by hormones in different periods including puberty and probably even in adult life. We know many locations in the brain where sex steroid hormones act to cause permanent structural changes. Nevertheless, the study of Phoenix, Goy, Gerall and Young still stands strong even after 50 years, confirming the revolutionary importance of their finding.

**Key words:** brain; sexual differentiation; steroid hormones; sex chromosomes

## Introduction

Men and women differ and we all know that. Males are usually larger, have hoarser voice, facial hair and more muscular body while females have breasts, lack facial hair and have usually so called feminine body with narrow waist and broader hips and chest. Of course, males and females also differ in appearance of their external and internal sexual organs. But do the differences end here? According to many studies performed in the last decades, we can now confidently say no. There are many other differences beside differences connected with sexual reproduction. Studies in recent years and decades have demonstrated differences in such diverse biological phenomena as wound healing (1), drug detoxication in liver (2), and perhaps most importantly, differences in the brain, which are no longer considered to be a myth but are believed to be present and are thought to be important for explaining many physiological and patophysiological processes occurring in our bodies (3). From clinical point of view it is very important to bear in mind that many different diseases show different prevalence between sexes, and these disparities could not be explained only by differences in lifestyle (what was initially suggested for the incidence of lung cancer). Many psychiatric disorders also show sex bias. For example, , major depressive disorder, anxiety

Received: 14 July 2009
Accepted for publication: 1 September 2009

and eating disorders are much more prevalent in women, while schizophrenia, autism and attention deficit disorder are diagnosed more often in men (4). Because of these clinical implications, studies of sex differences are not only of academic interest, but have important implications for clinical practice, which will, undoubtedly, became even more important in future years with the development of pharmacogenomics.

## Determination of sex in mammals

In mammals two sex chromosomes exist that determine the sex of the offspring. Females are homozygous for sex chromosomes with two X chromosomes and males are heterozygous with XY chromosomes. Sry, by far the smallest chromosome, poses an SRY gene, which is both sufficient and necessary for development of the male phenotype. SRY gene, first cloned in 1991 is small gene belonging to the group of high mobility group of proteins. Different studies have demonstrated that SRY alone is sufficient to trigger testis development (5, 6). Once testis is formed, hormones secreted from the testis govern subsequent development of male phenotype with antimullerian hormone (AMH/ MIS) being responsible for regression of female reproductive organs and steroid hormone testosterone being responsible for development of male secondary sexual organs. In females, ovaries remain relatively inactive until after birth and female secondary sexual organs develop in the absence of any hormonal exposure, what was clearly demonstrated by different clinical cases as well as in animal studies where even in complete absence of gonads (either ovary or testis) female secondary reproductive organs develop (7).

Reproductive development could be divided into two phases. Initial phase occurs during development in utero and comprises of gonadal differentiation and development of secondary sexual organs (penis, scrotum, accessory glands in males and clitoris, vagina, uterus and oviduct in females). This phase is followed by quiescent period during childhood. During puberty, second phase of sexual development occurs with hormones secreted from gonads (this time both from ovaries and testes) triggering sexual maturation and appearance of secondary sexual characteristics such as breasts and wide hips in females, facial hair, muscular body and hoarse voice in males. In addition, several recent studies have also shown that sexual hormones also influence brain development and that several changes occur in the brain during puberty due to exposure to large amount of sex steroid hormones (8).

## Development of sex differences in the brain

Brain control and govern all processes in the living organism, including reproduction. Therefore, it is not surprisingly or unexpectedly to know that sex differences exist also in the mammalian brain. This has been known for many decades with most differences being described in parts of limbic system, mostly in the hypothalamus and preoptic area, two areas closely connected with the function of the reproductive system (9). The classical view of brain sexual differentiation is built around the dogma that hormones secreted by gonads are solely responsible for differences in the brain between sexes. This hypothesis originated in 1959 when Phoenix, Goy, Gerall and Young published now classical study showing that prenatal administration of testosterone to female guinea pigs induced masculinized behavior in adult female guinea pigs (10). The importance of sex steroid hormones for differences in sexual behavior was acknowledged prior to this publication, although before 1959 it was believed that all actions of sex steroids are activational effects and not organizational. Study by Phoenix et al. (10) therefore for the first time showed that prenatal exposure to sex steroid testosterone could permanently alter brain function. Female guinea pigs that were given testosterone prenatally displayed masculinized behavior as adults, long after testosterone treatment, what could only resulted from permanent effect of testosterone on developing brain. Study by Phoenix et al. of course did not provide all important answers such as which hormone at what time and in what part of the brain is responsible for the sexual differentiation of the brain. Nevertheless, this study was of outmost importance as the first study showing that hormones could permanently alter brain structure and function. In the fifty years after this discovery, many questions about organizational effects of sex steroid hormones have been answered. We now know that at least in rodent brains, estradiol and not testosterone is responsible for the masculinization of the brain. Testosterone, secreted from the testes in male fetuses is transported into the brain, where it is converted into estradiol by cytochrome P450 aromatase, locally expressed in different parts of the brain (11, 12). While female fetuses are not exposed to testosterone from their gonads, they are still exposed to estradiol from their mothers. To prevent

masculinization of the female brain, large amounts of alpha-fetoprotein are present in the blood of female fetuses, which could bind estradiol and thus preventing it from entering into the brain (13). Studies in last decades have also indentified many areas of the brain that are altered during development due to exposure to sex steroids, not only areas closely connected with reproduction, but also in the areas important for emotional responses such as amygdala and even other areas such as hippocampus and cerebellum (14-17). One of the best known and studied examples is sexually dimorphic nucleus in the preoptic area (SDN), first identified by Gorski et colleagues in the late seventies (18). This nucleus is larger in males than in females and is believed to be important for male sexual behavior although its precise role is not yet known. SDN has been identified in different species such as sheep (19), macaque (20) and even humans (21, 22). Two other areas in the mammalian brain that are sexually dimorphic are ventromedial hypothalamic nucleus and bed nucleus of stria terminals (23-25). Both areas are involved in the regulation of sexual behavior and it is thus not surprisingly that these two areas are different in males and females. Perhaps more interesting are reports about sex differences in cerebellum and hippocampus (15-17). These two areas are not involved in the regulation of reproductive behavior, nevertheless, several studies have shown that sexual dimorphism exist also in hippocampus and cerebellum. Considering the function of these two areas, it is less surprisingly to find sex differences in morphology and gene expression. Hippocampus is considered to be involved in memory and spatial orientation, and spatial orientation in humans is now considered to be one of the important sexually dimorphic traits (26). As for cerebellum, several different human diseases such as autism and attention deficit disorder that show strong sexual dimorphism are thought to originate from the dysfunction of cerebellum (15). Therefore, it is not surprisingly to find sex differences also in these two areas.

## Is there a role for sex chromosomes in brain sexual differentiation?

Many studies in the last 50 years since the publication of the paper by Phoenix et al. (10) have shown the importance of sex steroid hormones for brain sexual differentiation. It is now clearly established that sex steroids have important role in brain development in different periods, not only prenatally but also postnatally, during puberty and in the adult life in both animals and humans. However, there was always a question lurking in the dark whether all sex differences in the brain could be explained by one unifying theory about organizational effects of sex steroids. The idea that sex chromosomes could also play a role in brain sexual differentiation was for sometime sidelined because some studies have shown that normal XX females could be completely masculinized (for some phenotypes) if treated with testosterone at appropriate time periods, and likewise, normal XY males could be completely feminized for some phenotypes if fetal testosterone production or action is blocked (27, 28). However, in the early nineties, several studies suggested that sex steroid hormones might not be the whole answer to sexual differentiation (29-32). In the last decade, several studies indeed provided evidence for hormone independent brain sexual differentiation.

Several approaches have been used to study sex differences in the brain that develop in the absence of hormone exposure. One approach is to study fetal brain development early during development, before gonads develop and start to produce sex steroid hormones. This approach was used in several studies and has provided evidence that some sex differences do occur very early during development, before fetuses are exposed to endogenous sex steroid hormones. Study by Kolbinger et al. (30) demonstrated sex differences in dopaminergic neurons in rat fetuses already on day 14.5 p.c. while genomic study by Dewing et al. (33) identified over 50 genes whose expression differed between male and female mouse brain on day 10.5 p.c., well before gonads start to produce sex steroids. However, of real importance would be studies that would demonstrate hormone independent sex differences in adult animals, either in brain morphology or behavior. To achieve these goals, two different models, each with advantages and disadvantages, have been developed.

A very useful model for studying genetic differences between sexes is so called four core genotype (FCG) mouse model. In these mice, *sry* gene has been manipulated (translocated or mutated) to produce normal XY males, normal XX females, XX males (*sry* gene translocated to autosome) and XY females (*sry* gene mutated) (34). In XY females and XX males genetic sex does not correspond with phenotypic sex and therefore, relative contribution of sex chromosomes and sex hormones could be studied. This is the most studied model for hormone independent brain sexual differentiation so far, and

several studies have shown some differences that could not be attributed to sex hormones but must arise due to differences in sex chromosomes. Initial studies with FCG mice did not reveal any differences that could be attributed to the effect of sex chromosomes for different parameters such as male sexual behavior, cell numbers in hypothalamic anteroventral periventricular nucleus (AVPV), the size of the spinal nucleus of the blubocavernosus (SNB), cortical thickness and progesterone receptor expression in preoptic area (POA) (35, 36). These studies therefore confirmed classical organizational-activational-hypothesis of brain sexual differentiation. However, arginin vasopressin (AVP) immunoexpression in lateral septum (LS), which is also known to be sexually dimorphic, differed between XY and XX mice of the same phenotypic sex suggesting that this difference is partially dependent on sex chromosomes (34, 37). Further studies revealed even stronger evidence that sex chromosomes do account for some sex differences between male and female mice. When mesencephalic cells were dissociated from 14.5 days old mouse embryos and cultured, more dopamine producing cells (I.E. tyrosine hydroxlase expressing cells) developed in cultures from XY embryos than in those from XX embryos (38), what confirmed the results from previous studies (30, 31). Adult FCG mice were also tested for male to male aggressive behavior (after testosterone treatment of adult gonadectomized mice) and XY females were more aggressive than XX females while there was no difference between XY and XX males (37). Furthermore, there were differences in nociception, parental behavior and habit formation that could not be attributed to sex hormones but have to be consequences of sex chromosomes (39-42). Perhaps most interestingly, FCG mouse model was also applied to studies of incidence and progression of autoimmune diseases, a very important issues as most autoimmune diseases in humans including multiple sclerosis and systemic lupus erhytematosus have a strong sex difference in prevalence. XX mice showed much stronger autoimmune responses than XY mice and although organizational/activational effect of sex steroid hormones do account to some extent for observed sex differences, a study by Smith-Bouvier et al. (43) strongly suggest that sex chromosomes also play an important role in development of the differences between sexes in incidence and progression of autoimmune diseases.

Steroidogenic factor 1 (SF-1) was initially discovered as a transcription factor regulating expression of different steroidogenic enzymes (44). Further studies, however, revealed it's much wider role in development and function of endocrine system as SF-1 knockout mice are born without gonads and adrenal glands, have disorganized ventromedial hypothalamic nucleus and unfunctional gonadotrope cells in the pituitary (45, 46). In SF-1 knockout mouse embryos, genital ridges form normally on day 10.5 p.c. (46). However, almost immediately after formation of genital ridges, cells became apoptotic and by day 12.5 p.c., genital ridges disappear. As steroidogenesis in fetal mouse testis starts only after day 12.5 p.c., these mice are never exposed to any endogenous sex steroid hormones. SF-1 knockout mice are born completely sex reversed; both XX and XY pups show female phenotype. Since SF-1 knockout mice are never exposed to any sex steroid hormones, they are another very useful model to study hormone independent development of sex differences in the brain. SF-1 knockout model differ from FCG model in one very important way: FCG mice develop gonads independently from chromosomal sex and are thus exposed to sex steroid hormones during neonatal and pubertal development. Sex steroid hormones could influence brain development and could perhaps even mask or overcome some sex differences that would develop in complete absence of hormones. SF-1 knockout mice are, in contrast to FCG mice, never exposed to any endogenous sex steroid hormones and thus provide a unique model allowing searching for sex differences that develop in true hormone-less environment. Initial studies with SF-1 knockout mice, like studies with FCG mice, did not reveal any major differences between sexes. As expected, sexually dimorphic nucleus was not present in either XX or XY SF-1 knockout mice, conforming that prenatal exposure to testosterone is necessary for the development of this nucleus. However, immunocytochemical studies did reveal some sex differences present in both WT and SF-1 knockout mice such as number of calbindin immunopositive cells in the ventromedial hypothalamus and neural nitric oxide synthase in the AVPV (47). However, sex difference in AVP expression in LS was not confirmed, suggesting that other factors and not just sex chromosomes influence expression of AVP in LS. Recent studies with SF-1 knockout mice revealed very interesting observation in female sex behavior. Unlike in rats, WT mice of both sexes are capable of showing female sexual behavior when treated with estradiol and progesterone. In our studies we found that although mice from all four

(WT male, WT female, SF-1 knockout male, SF-1 knockout female) groups did show lordosis, there was a large difference in lordosis quotient between WT male and female mice, with, as expected, female mice showing much stronger lordotic response when stimulated by a WT stud male. SF-1 knockout mice of both sexes also showed lordosis, although it was not as strong as in WT females, suggesting that developmental exposure to sex steroids is important also for proper development of lordotic behavior in adult mice. However, most interestingly, there was a significant sex difference in lordosis quotient between XX and XY SF-1 knockout mice suggesting that this behavioral trait is at least partially influenced by sex chromosomes. Similarly to FCG mice, small sex differences were also found in parental and some social behaviors between XX and XY SF-1 knockout mice, suggesting the effect of sex chromosomes.

## Conclusions

Many decades of studies have convincingly shown that differences between male and female brain exist. Undoubtedly, many studies have demonstrated morphological differences between male and female brains in animals, and some studies have provided evidence that such differences most likely exist also in humans. We do not understand all the processes that govern sexually dimorphic brain development and several recent studies suggested that sex chromosomes, not only sex hormones, could influence sex specific development. More difficult are questions how to correlate morphological differences in the brain with certain sex specific behaviors, although even there we saw a big progress in recent years. Several studies have provided evidence that sex differences in hippocampus might be connected with sex differences in spatial orientation, and sex differences in amygdala might be connected with differences in emotional responses. Since the seminal paper by Phoenix and colleagues in 1959, we have made large strides ahead and we now have answers to many questions, asked by Phoenix and colleagues. Nevertheless, many questions still remain unanswered and are waiting for new studies to shed the light.

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## SE MOŠKI MOŽGANI RAZLIKUJEJO OD ŽENSKIH?

G. Majdič

**Povzetek:** Leta 1959, natančno pred petdesetimi leti, je bil objavljen članek z naslovom "Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig" (Organizacijski vpliv testosterona pred rojstvom na tkiva, ki urejajo spolno obnašanje pri samicah morskih prašičkov) avtorjev Phoenixa, Goya, Geralla in Younga. Pred objavo tega članka je splošno veljalo, da spolni hormoni lahko vplivajo na delovanje možganov, a obstajalo je prepričanje, da hormoni lahko vplivajo na možgane samo trenutno, tako da sprožijo določene procese kot je na primer spolno obnašanje. V nasprotju s tem prepričanjem so Phenix in sodelavci prvič pokazali dokaze, da spolni hormoni lahko dolgoročno spremenijo strukturo možganov in tako povzročijo razlike v obnašanju, ki se pokažejo šele dolgo časa po dejanskem delovanju spolnih hormonov. Raziskava Phoenixa in sodelavcev je bila zato revolucionarna, saj je postavila popolnoma novo dogmo, in zato je pričakovano imela tudi veliko nasprotnikov. Tudi sami avtorji so bili previdni, saj niso imeli odgovorov na številna vprašanja. Zato nikjer niso neposredno trdili, da hormoni zares lahko vplivajo na strukturo možganov ali centralnega živčnega sistema, temveč so raje uporabljali izraz "tkiva, ki sodelujejo pri urejanju spolnega obnašanja". Seveda je tudi ta raziskava, kot mnoga druga revolucionarna odkritja, pustila več vprašanj kot pa podala odgovorov. Avtorji te raziskave niso mogli vedeti, kdaj in kje natančno spolni hormoni vplivajo na razvoj možganov. Prav tako niso vedeli, ali na možgane vpliva neposredno testosteron ali kakšen njegov presnovni produkt. V petdesetih letih od objave članka Phoenixa in sodelavcev je bilo objavljenih na desetine ali celo stotine raziskav, ki so potrdile osnovna opažanja avtorjev in odgovorile na mnoga vprašanja. Danes tako vemo, da je vsaj pri glodavcih ženski spolni hormon estradiol (ki nastane iz testosterona lokalno v možganih) tisti, ki zares sproži razvoj moških možganov. Vemo tudi, da se lahko možgani spreminjajo pod vplivom spolnih hormonov v različnih obdobjih, pred rojstvom in po njem, v času pubertete in najverejtneje do neke mere tudi v odraslem življenju. Vseeno pa je raziskava Phoenixa, Goya, Geralla in Younga tudi po petdesetih letih še vedno aktualna in veljavna, kar potrjuje revolucionarnost njihovega odkritja.

**Ključne besede:** možgani; spolne razlike; steroidni hormoni; spolni kromosomi

Slov Vet Res 2009; 46 (3): 93-103
UDC 636.7.09:612.1:547.567:66.094.3.097.8

**Original Research Paper**

# 24-HOUR FOLLOW-UP STUDY OF PLASMA COENZYME $Q_{10}$, TOTAL ANTIOXIDANT CAPACITY AND SELECTED BLOOD PARAMETERS AFTER A SINGLE ORAL DOSE OF WATER-SOLUBLE COENZYME $Q_{10}$ IN HEALTHY BEAGLE DOGS

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**Summary:** Coenzyme Q ($CoQ_{10}$) is one of the most promising compounds in antioxidant therapy, due to its key role in mitochondria and its antioxidant action. It has been shown to have positive effects in the treatment of many diseases in humans. In contrast, there are only a limited number of studies and experimental data on $CoQ_{10}$ supplementation in dogs. In the present study, changes of plasma $CoQ_{10}$, serum total antioxidant capacity (TAC), and selected haematological and biochemical parameters were followed over 24 hours in healthy beagle dogs, following the administration of a single oral dose of 30 mg of water-soluble $CoQ_{10}$. Correlations between plasma $CoQ_{10}$ and serum TAC, and between TAC and albumin, a potent plasma antioxidant, were also investigated. A statistically significant increase of plasma $CoQ_{10}$ was demonstrated as early as 20 minutes after $CoQ_{10}$ administration, with a peak value four hours after administration. Contrary to expectation, simultaneous increases of plasma $CoQ_{10}$ and TAC were not observed, however, a positive, statistically significant correlation between the latter two parameters was observed one hour after the administration of $CoQ_{10}$ ($p = 0.011$), indicating a contribution of $CoQ_{10}$ to the TAC of canine serum. The correlation, now close to statistical significance ($p = 0.052$), remained when plasma $CoQ_{10}$ reached its peak value. The positive, statistically significant correlations between albumin and serum TAC, determined at basal measurements and 40 minutes and 1, 4 and 6 hours later, indicate a significant contribution of albumin to the TAC of canine serum. Selected haematological and biochemical parameters over 24 hours showed the diurnal variations normally found in dogs. Further investigation is needed to establish the influence of long-term $CoQ_{10}$ supplementation on TAC in dogs.

**Key words:** coenzyme $Q_{10}$; total antioxidant capacity; dogs

## Introduction

Coenzyme Q (CoQ) or ubiquinone is an endogenous compound located widely in living organisms. It is a lipid, composed of a redox active benzoquinone ring and a hydrophobic side chain comprising from 6 ($CoQ_6$) to 10 ($CoQ_{10}$) isoprenoid units, depending on species (1, 2). In humans and most mammals, including dogs, the predominant form is $CoQ_{10}$, which is the same as that available as an oral formulation (1, 3). $CoQ_{10}$ is present in two redox forms, namely ubiquinone-10 ($CoQ_{10}$, oxidized form) and ubiquinol-10 ($CoQ_{10}H_2$, reduced form). The latter is the predominant form in blood and most other tissues, where it behaves as a phenolic antioxidant (1, 4, 5, 6, 7, 8).

CoQ has several biochemical functions. As an intermediate of the electron transport system in mitochondria it plays a key role in cellular respiration and production of adenosine triphosphate (ATP). The reduced form of CoQ, ubiquinol, is the only known lipid-soluble antioxidant synthesized *de novo* in human and all animal tissues, and mechanisms exist that can generate it from ubiquinone as

Received: 23 February 2009
Accepted for publication: 31 August 2009

a result of its antioxidant activity. Its strong hydrophobicity allows the insertion of the molecule into the membrane phospholipid bilayer in proximity to the unsaturated lipid chains, where it acts as a primary scavenger of free radicals and thus prevents lipid peroxidation in most subcellular membranes. The protective effect of ubiquinol includes not only lipids, but extends to proteins and DNA (1, 2, 5, 9, 10-14). In addition to its direct antioxidant function, ubiquinol regenerates α-tocopherol by reducing the α-tocopheryl radicals produced by reaction with lipid or oxygen (1, 11, 15), and is responsible for the extracellular stabilization of ascorbate with its NADH-dependent reductase (16).

There is also evidence for a function of CoQ in redox control of cell signalling and gene expression, from studies on coenzyme Q stimulation of cell growth, inhibition of apoptosis, control of thiol groups, formation of hydrogen peroxide and control of membrane channels (11, 12, 17, 13).

$CoQ_{10}$ is emerging as prophylactic and therapeutic agent. It is one of the most promising compounds in antioxidant therapy, due to its key role in mitochondria and antioxidant action (1, 4, 5, 10, 11, 13, 18). In human studies $CoQ_{10}$ has been shown to be a valuable component in treating cardiovascular (1, 4, 19, 20), neurodegenerative (1, 9, 21, 22) and renal diseases (23, 24), as well as male infertility (4, 9, 25) and cancer (9, 26). It is also able to inhibit oxidative damage, to enhance DNA repair enzyme activity in human cultured lymphocytes (27) and to prevent many of the detrimental effects of photoaging on the skin (28).

Although there are some basic similarities in the function of $CoQ_{10}$ in humans and other animals, only a limited number of studies and experimental data about $CoQ_{10}$ supplementation have been reported on dogs. So far, $CoQ_{10}$ has been used as a supportive therapy for cardiac and hepatic diseases, in mitochondrial diseases and as a neuroprotectant in dogs (6, 29, 30, 31).

The aim of this 24-hour follow-up study was to determine changes of plasma $CoQ_{10}$, serum total antioxidant capacity (TAC) and selected blood parameters in healthy beagle dogs, following a single oral dose of water-soluble $CoQ_{10}$. The study was also aimed to determine whether there is a correlation between plasma $CoQ_{10}$ and serum TAC. The reduced form of $CoQ_{10}$, the predominant form in plasma and tissues, exerts antioxidant properties and TAC is a biochemical parameter suitable for evaluating the overall antioxidant status of serum or plasma resulting from antioxidant intake and/or production, and their consumption by the normal or increasing levels of oxidative stress (32-36). In addition, correlations between albumin and serum TAC were determined at each sampling time. Albumin is the predominant circulating antioxidant agent, since each albumin molecule contains one single cysteine with a free SH group that participates in redox reactions. Furthermore, albumin, urate and ascorbate make up the major contribution to the TAC of human plasma, largely due to their high concentrations relative to those of other blood antioxidants such as bilirubin, α-tocopherol, β-carotene, glutathione, ubiquinol-10, as well as those not yet recognized (34-38).

## Materials and methods

### *Animals*

7 adult beagle dogs, 1 female and 6 male, weighing between 16.5 and 22.6 kg with an average body weight of 19.5 kg, were used in this study. They were considered healthy on the basis of history, results of physical examination and of haematological and serum biochemical analysis,. The dogs were housed in couples in cages of appropriate size in a room with room temperature between 18 and 21°C, fed a commercial dry and canned diet (Pedigree Pal, Mars Incorporated, USA) three times a day, with unlimited access to water. They were walked in pairs for at least 20 minutes three times per day. Social contacts between the caretakers and dogs were carried out during the day.

All procedures complied with the relevant Slovenian governmental regulations (Animal Protection Act UL RS, 43/2007) and were approved by Ministry of Agriculture, Forestry and Food, Veterinary Administration of the Republic of Slovenia; license No 323-02-818/2005.

### *Study protocol and collection of blood samples*

Each dog received a single dose of water soluble paste containing 7.5 % of $CoQ_{10}$ in the form of an inclusion complex with β-cyclodextrin, that was synthesized in the Laboratory for Food Chemistry, National Institute of Chemistry (Ljubljana, Slovenia) according to previously filed patents (39, 40). 400 mg of paste, equivalent to 30 mg of $CoQ_{10}$, was added into the food and given with the morning meal (Pedigree Pal, Mars Incorporated, USA) at 8 a.m. Ve-

nous blood samples for determination of $CoQ_{10}$, total antioxidant capacity (TAC), and haematological and biochemical parameters were collected before (basal values), and 20 and 40 minutes, 1, 2, 4, 6, 10 and 24 hours after dosing. The dogs were fed three times a day, at 8 a.m., 2 p.m. (6 h after dosing) and at 6 p.m. (10 h after dosing), each time after blood was collected. Water was available *ad libitum*.

Blood samples for $CoQ_{10}$ were collected in heparinized Vacutainer® tubes (Vacutainer Systems, Becton Dickinson, Franklin Lakes, New Jersey, USA) and immediately centrifuged at 1500 g for 15 minutes at 4°C. Plasma was separated and immediately frozen at -80°C until analysis. Blood samples were collected into serum separator tubes (Vacuette, Greiner Bio-One, Kremsmunster, Austria) and into EDTA-containing tubes (Mictrotainer™, Beckton and Dickinson, Franklin Lakes, USA). Samples in serum separator tubes were stood for 30 min at 4°C to clot, then centrifuged (1300 g for 10 min) to separate the serum. Serum samples were stored at -80°C and assayed in duplicate within 2 weeks for TAC and various biochemical parameters including glucose (Glu), urea, creatinine (Crea), sodium (Na), potassium (K), chloride (Cl), calcium (Ca), inorganic phosphate (iP), total protein (TP), albumin (Alb), alanine aminotransferase (ALT) and alkaline phosphatase (AP). EDTA blood samples for complete blood count (CBC) and white cell differential count (WCDC) determinations were stored at room temperature and analysed between 1 and 5 h after sampling.

### *Plasma $CoQ_{10}$ determination*

Plasma $CoQ_{10}$ was determined at the National Institute of Chemistry (Ljubljana, Slovenia) by HPLC/MS as previously described in a bioavailability study of water-soluble $CoQ_{10}$ in dogs (41). Plasma samples (400 µL) were denatured with 200 µL of 10% perchloric acid in ethanol (v/v) and extracted three times with 2 mL of n-hexane. The combined organic extracts were concentrated with a rotary evaporator (Rotavapor R-144 equipped with a water bath B-480, Büchi, Flawil, Switzerland). The residue was redissolved in 200 µL of 2-propanol and analyzed by HPLC/MS.

### *Determination of TAC*

Serum samples from 7 healthy beagle dogs were assayed for TAC using an automated chemistry analyser (RA-XT, Siemens/Bayer (former Technicon), Munich, Germany), using a commercially available Total Antioxidant Status (TAS) kit (Randox, Crumlin, UK), following the manufacturer's instructions. The assay (32) is based on the reduction of free radicals ($ABTS^{*+}$-2,2'–azinobis-(3-ethylbenzothiazoline-6-sulfonate)) by antioxidants, measured as a decrease of absorbance at 600 nm at 3 min. The $ABTS^{*+}$ radical cation is formed by the interaction of ABTS with the ferrylmyoglobin radical generated by the activation of metmyoglobin with hydrogen peroxide. The suppression of the absorbance of the $ABTS^{*+}$ radical cation by serum antioxidants was compared with that by Trolox (6-hydroxy-2,5,7,-tetramethylchroman-2-carboxylic acid), which is included as part of the TAS kit. The results are expressed as mmol/l of Trolox equivalents.

### *Determination of biochemical parameters*

Glucose, urea, creatinine, calcium, inorganic phosphate, total protein, albumin, AP and ALT were determined by automated chemistry analyser (RA-XT, Siemens/Bayer (former Technicon), Munich, Germany). Electrolytes, Na, K and Cl, were determined by electrolyte analyser Ilyte Na/K/Cl (Instrumentation Laboratory, Lexington, MA, USA).

### *Determination of haematological parameters*

CBC and WCDC were determined by an automated laser haematology analyser H*1 (Siemens/Bayer (former Technicon), Munich, Germany) with species specific software (H*1 Multi-Species V30 Software, Tarrytown, New York, USA). The resulting CBC includes white blood cells (WBC), red blood cells (RBC), haemoglobin concentration (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and platelets (PLT). WCDC comprises six-part differential: neutrophils (NEUT), lymphocytes (LYMPH), monocytes (MONO), eosinophils (EOS), basophils (BASO) and large unstained cells (LUC), all as percentages. The LUC category consists of a heterogeneous population of all large cells that fail to exhibit any peroxidase activity (atypical lymphocytes, immature granulocytes and blasts).

### *Statistical evaluation*

Data were analysed with commercial software (SPSS 15.0, Chicago, Illinios, USA). For each param-

eter measured the data were examined for normality using the Kolmogorov and Smirnov test (42). Means and standard deviations (SD) were calculated for plasma $CoQ_{10}$, serum TAC and selected biochemical and haematological parameters. Repeated measures ANOVA was used to test for statistically significant differences of parameters between basal samples and 8 consecutive samples within the 24-hour measuring period. Pearson's correlation coefficient analysis was performed to determine whether there were statistically significant correlations between plasma $CoQ_{10}$ and serum TAC and between albumin and serum TAC at different sampling times. A value of $p < 0.05$ was considered significant.

## Results

Administration of a single oral dose of water-soluble $CoQ_{10}$ to healthy beagle dogs resulted in a significant increase in plasma $CoQ_{10}$ (Table 1) as early as 20 min after basal measurements, and remained significantly increased during the rest of the measuring period. Plasma $CoQ_{10}$ reached a peak value of 1.21 ± 0.48 mg/L, four hours after the basal sampling that gave a mean endogenous plasma $CoQ_{10}$ value of 0.36 ± 0.09 mg/L. After reaching the peak concentration, $CoQ_{10}$ decreased continuously from the 6$^{th}$ to the 24$^{th}$ hour after administration to a final value of 0.70 ± 0.17 mg/L.

Serum TAC (Table 1) decreased significantly 2 hours after basal sampling, from 0.948 ± 0.240 to 0.674 ± 0.132 mmol/L. It then increased from the 4$^{th}$ to the 24$^{th}$ hour after $CoQ_{10}$ administration to a final value of 0.986 ± 0.152 mmol/L.

Albumin concentration (Table 1) remained within the normal reference range (43,44) at all sampling times without significant changes from basal values.

A positive, statistically significant correlation (Table 1) was found between plasma $CoQ_{10}$ and serum TAC one hour after basal sampling ($r = 0.869$; $p = 0.011$). Four hours after basal measurements, when plasma $CoQ_{10}$ had reached its peak value, a positive correlation close to statistical significance ($r = 0.808$; $p = 0.052$) was determined. Though not significant, nearly all correlations at other sampling times were positive, with the exception of a negative correlation found at 10 hours after the administration of $CoQ_{10}$.

A positive, significant correlation between albumin and serum TAC (Table 1) was determined at basal sampling and 40 minutes, 1, 4 and 6 hours later. Correlations at the remaining time points were also positive.

All serum biochemical parameters, with the exception of AP and albumin, showed statistically significant changes from basal values at all sampling times of the 24-hour measuring period (Table 2). Basal measurements showed that, with the exception of AP and inorganic phosphate, all other biochemical parameters were within the normal reference range (43, 44). At other sampling times however, biochemical parameters showed minor deviations from the normal reference range.

Haematological parameters, with the exception of LUC, differed statistically significantly from basal values throughout the 24-hour measuring period (Table 3). All basal haematological values were within the normal reference range (43, 45). On the other hand there were minor deviations of MCH, MCHC, NEUT and LYMPH from normal values at other sampling times of the 24-hours measuring period.

## Discussion

A limited number of studies and experimental data about $CoQ_{10}$ supplementation in different physiological and pathological conditions in dogs are available in the literature (30, 31, 41, 46, 47). In contrast, there is a great interest and progress in this area of human medicine, where numerous studies have confirmed that $CoQ_{10}$, due to its key role in mitochondria and antioxidant action (1, 5, 9, 10, 11, 13), is one of the most promising compounds in antioxidant therapy of cardiovascular (1,4, 19, 20), neurodegenerative (1, 9, 21, 22), renal (23, 24) and immune diseases (27), as well as in male infertility (4,9, 25), cancer (9, 26) and several other disorders.

In the present study, changes of plasma $CoQ_{10}$, serum TAC and selected haematological and biochemical parameters were followed over 24 hours in healthy beagle dogs after administration of a single oral dose of water-soluble $CoQ_{10}$. The reduced form of $CoQ_{10}$, ubiquinol-10, is an antioxidant and also a predominant form of $CoQ_{10}$ in human and canine plasma and tissues (1, 4, 5, 6, 7, 8), therefore, in our study we aimed to determine whether there is a correlation between plasma $CoQ_{10}$ and serum TAC at different sampling times. Correlations between albumin and serum TAC were also determined, since the antioxidant properties of albumin and its contribution to the TAC of human plasma are well known (32, 34-38).

**Table 1:** Plasma $CoQ_{10}$, serum TAC and albumin concentration (mean ± SD) and correlations between plasma $CoQ_{10}$ and serum TAC and between albumin and serum TAC at different sampling times within a 24-hour measuring period

| Parameter/Time of sampling | Basal value | 20 min | 40 min | 1 h | 2 h | 4 h | 6 h | 10 h | 24 h |
|---|---|---|---|---|---|---|---|---|---|
| $CoQ_{10}$ (mg/L) | 0.36 ± 0.09 | 0.45 ± 0.10* | 0.51 ± 0.07* | 0.61 ± 0.10* | 1.00 ± 0.37 * | 1.21 ± 0.48* | 0.88 ± 0.27* | 0.77 ± 0.18* | 0.70 ± 0.17* |
| TAC (mmol/L) | 0.948 ± 0.240 | 0.814 ± 0.252 | 0.844 ± 0.114 | 0.820 ± 0.131 | 0.674 ± 0.132 * | 0.804 ± 0.124 | 0.868 ± 0.090 | 0.862 ± 0.073 | 0.986 ± 0.152 |
| Pearson correlation coefficient (r)[a] | 0.451 | 0.044 | 0.540 | 0.869 | 0.164 | 0.808 | 0.477 | -0.068 | 0.473 |
| p value[a] | 0.309 | 0.926 | 0.211 | **0.011¤** | 0.793 | **0.052** | 0.279 | 0.898 | 0.510 |
| Alb (g/L) | 34.5 ± 3.8 | 35.1 ± 4.1 | 34.0 ± 4.1 | 34.7 ± 3.9 | 35.4 ± 4.2 | 34.4 ± 3.6 | 34.8 ± 3.8 | 35.1 ± 3.9 | 34.7 ± 3.9 |
| Pearson correlation coefficient (r)[b] | 0.792 | 0.547 | 0.935 | 0.815 | 0.678 | 0.972 | 0.837 | 0.206 | 0.466 |
| p value[b] | **0.034¤** | 0.204 | **0.002¤** | **0.025¤** | 0.138 | **0.001¤** | **0.019¤** | 0.696 | 0.292 |

* $p < 0.05$ in comparison with basal values
¤ statistically significant correlation
[a]correlations between plasma $CoQ_{10}$ and serum TAC
[b]correlations between albumin and serum TAC

Table 2: Selected biochemical parameters (mean ± SD) at different sampling times within a 24-hour measuring period

| Parameter/Time of sampling | Basal value | 20 min | 40 min | 1 h | 2 h | 4 h | 6 h | 10 h | 24 h |
|---|---|---|---|---|---|---|---|---|---|
| Glu (mmol/L) | 5.10 ± 0.36 | 5.72 ± 0.29 * | 5.71 ± 0.14 * | 5.68 ± 0.33 * | 5.95 ± 0.32 * | 5.70 ± 0.17 * | 5.47 ± 0.18 * | 6.00 ± 1.07 | 5.22 ± 0.16 |
| Urea (mmol/L) | 6.54 ± 1.74 | 6.11 ± 1.77 | 6.17 ± 1.83 | 6.57 ± 1.82 * | 6.75 ± 2.10 * | 6.29 ± 2.04 | 5.90 ± 1.87 * | 6.48 ± 1.49 | 6.04 ± 1.64 |
| Crea (µmol/L) | 71.5 ± 15.7 | 71.7 ± 16.2 | 68.0 ± 13.8 | 60.6 ± 13.2 * | 78.7 ± 28.0 | 68.2 ± 22.9 | 62.7 ± 21.9 | 55.5 ± 14.8 | 71.3 ± 15.1 |
| Na (mmol/L) | 148.0 ± 1.0 | 147.8 ± 0.8 | 147.2 ± 0.7 | 146.9 ± 0.6 * | 146.9 ± 0.9 * | 148.3 ± 0.5 | 147.0 ± 0.8 * | 148.4 ± 1.6 | 148.3 ± 0.9 |
| K (mmol/L) | 5.32 ± 0.37 | 4.92 ± 0.40 * | 4.74 ± 0.34 * | 4.79 ± 0.13 * | 4.99 ± 0.28 | 4.82 ± 0.20 * | 5.00 ± 0.31 * | 5.09 ± 0.44 | 4.87 ± 0.32 * |
| Cl (mmol/L) | 113.5 ± 1.4 | 113.3 ± 1.8 | 112.6 ± 1.9 | 112.7 ± 1.2 | 111.6 ± 1.6 * | 110.7 ± 1.9 * | 110.5 ± 1.5 * | 112.4 ± 2.5 | 111.4 ± 2.0 * |
| Ca (mmol/L) | 2.40 ± 0.12 | 2.36 ± 0.13 | 2.30 ± 0.11 | 2.54 ± 0.17 | 2.78 ± 0.43 | 2.84 ± 0.44 * | 2.51 ± 0.18 | 2.91 ± 0.54 | 2.44 ± 0.11 |
| iP (mmol/L) | 1.74 ± 0.21 | 1.62 ± 0.14 * | 1.63 ± 0.13 | 1.95 ± 0.10 * | 2.32 ± 0.31 * | 1.98 ± 0.29 | 1.65 ± 0.14 | 2.01 ± 0.30 | 1.59 ± 0.15 |
| AP (U/L) | 217.9 ± 173.4 | 216.1 ± 171.2 | 214.4 ± 170.2 | 214.4 ± 172.1 | 212.2 ± 170.5 | 229.4 ± 186.3 | 228.1 ± 183.7 | 213.4 ± 175.1 | 221.1 ± 180.6 |
| ALT (U/L) | 72.5 ± 34.4 | 78.1 ± 35.8 | 74.6 ± 35.0 | 89.4 ± 36.7 * | 78.0 ± 33.0 | 81.5 ± 35.2 | 78.8 ± 36.1 | 80.9 ± 32.8 | 73.8 ± 33.3 |
| TP (g/L) | 67.6 ± 3.6 | 67.7 ± 3.2 | 66.0 ± 2.8 | 72.5 ± 3.7 * | 80.1 ± 8.1 * | 72.6 ± 7.6 | 68.2 ± 4.5 | 75.8 ± 12.7 | 66.7 ± 3.1 |

* $p < 0.05$ in comparison with basal values

Table 3: Haematological parameters (mean ± SD) at different sampling times within a 24-hour measuring period

| Parameter/Sampling time | Basal value | 20 min | 40 min | 1 h | 2 h | 4 h | 6 h | 10 h | 24 h |
|---|---|---|---|---|---|---|---|---|---|
| WBC ($x10^9$/L) | 10.55 ± 2.66 | 10.50 ± 2.84 | 10.74 ± 3.03 | 10.65 ± 2.87 | 11.09 ± 3.25 | 10.38 ± 3.17 | 10.00 ± 3.00 | 10.44 ± 2.86 | 9.47 ± 2.55 * |
| RBC ($x10^{12}$/L) | 6.114 ± 0.658 | 5.957 ± 0.681 | 6.008 ± 0.664 | 6.002 ± 0.708 | 5.754 ± 0.724 * | 5.635 ± 0.594 * | 5.892 ± 0.713 | 5.977 ± 0.586 | 5.862 ± 0.803 |
| HGB (g/L) | 147.1 ± 14.9 | 142.7 ± 15.4 * | 144.0 ± 14.6 | 144.7 ± 15.5 * | 142.2 ± 15.4 * | 136.7 ± 12.6 | 140.7 ± 15.5 | 146.8 ± 12.3 | 139.7 ± 16.2 |
| HCT (l/L) | 0.410 ± 0.039 | 0.400 ± 0.040 | 0.404 ± 0.040 | 0.407 ± 0.041 | 0.388 ± 0.041 * | 0.380 ± 0.035 * | 0.391 ± 0.042 | 0.435 ± 0.035 * | 0.388 ± 0.048 |
| MCV (fL) | 67.11 ± 1.68 | 67.14 ± 1,48 | 67.28 ± 1.57 | 67.51 ± 1.49 | 67.56 ± 1.64 * | 67.50 ± 1.43 | 66.44 ± 1.71 * | 73.05 ± 1.71 * | 66.51 ± 1.37 * |
| MCH (pg) | 24.08 ± 0.71 | 23.98 ± 0.58 | 23.97 ± 0.69 | 24.12 ± 0.61 | 24.78 ± 1.04 * | 24.24 ± 0.94 | 23.90 ± 0.59 | 24.58 ± 0.72 * | 23.87 ± 0.58 |
| MCHC (g/L) | 358.8 ± 5.2 | 357.5 ± 3.9 | 356.4 ± 3.4 | 357.4 ± 4.6 | 366.7 ± 8.2 * | 359.2 ± 8.2 | 359.7 ± 3.3 | 336.4 ± 5.9 * | 359.0 ± 2.4 |
| PLT ($x10^9$/L) | 418.7 ± 79.2 | 397.7 ± 71.5 * | 401.4 ± 66.4 | 403.8 ± 73.4 | 442.2 ± 77.6 * | 429.0 ± 63.8 | 403.0 ± 68.4 | 403.0 ± 65.2 | 393.5 ± 56.6 |
| NEUT (%) | 64.47 ± 4.90 | 64.27 ± 4.13 | 64.17 ± 3.95 | 64.68 ± 4.31 | 61.61 ± 4.15 * | 61.56 ± 5.78 | 60.70 ± 4.53 * | 58.52 ± 3.46 * | 58.38 ± 5.55 * |
| LYMPH (%) | 30.27 ± 4.54 | 30.32 ± 3.83 | 30.41 ± 3.52 | 29.88 ± 4.04 | 32.54 ± 4.29 * | 32.50 ± 4.87 | 33.54 ± 4.39 * | 32.47 ± 2.88 | 35.75 ± 5.21 * |
| MONO (%) | 2.257 ± 0.310 | 2.400 ± 0.597 | 2.129 ± 0.499 | 2.229 ± 0.811 | 2.600 ± 0.914 | 2.400 ± 0.391 | 1.986 ± 0.414 | 3.414 ± 0.931 * | 2.071 ± 0.878 |
| EOS (%) | 2.657 ± 0.939 | 2.671 ± 0.834 | 2.814 ± 0.859 | 2.757 ± 0.896 | 2.729 ± 0.763 | 3.086 ± 1.027 | 3.414 ± 0.773 * | 5.171 ± 1.361 * | 3.429 ± 1.095 * |
| BASO (%) | 0.114 ± 0.900 | 0.129 ± 0.049 | 0.157 ± 0.054 | 0.157 ± 0.079 | 0.157 ± 0.054 | 0.143 ± 0.054 | 0.129 ± 0.049 | 0.186 ± 0.069* | 0.143 ± 0.054 |
| LUC (%) | 0.200 ± 0.0816 | 0.229 ± 0.0951 | 0.371 ± 0.160 | 0.286 ± 0.122 | 0.357 ± 0.162 | 0.300 ± 0.163 | 0.257 ± 0.113 | 0.229 ± 0.170 | 0.214 ± 0.900 |

* $p < 0.05$ in comparison with basal values

The administration of $CoQ_{10}$ resulted in a significant increase in plasma $CoQ_{10}$ concentration as early as 20 minutes after basal measurements, reaching a peak value of 1.21 ± 0.48 mg/L at four hours, then slowly decreasing until the final measurement at 24 hours, as already reported in a bioavailability study of water soluble $CoQ_{10}$ (41). These results are in agreement with those of a bioavailability study of oral $CoQ_{10}$ formulations (46), in which dogs received 60 mg (2 x 30 mg) of $CoQ_{10}$ in three different formulations. The mean endogenous plasma $CoQ_{10}$ in dogs prior to oral administration of $CoQ_{10}$ was in general agreement with our basal values of $CoQ_{10}$, 0.21 ± 0.07 mg/L and 0.36 ± 0.09 mg/L, respectively. Basal plasma $CoQ_{10}$ values in the present study were lower than the serum $CoQ_{10}$ values of the control group of dogs in a study of Harker-Murray et al.(31). The difference is presumably due to different types of sample used and methods of measurements applied.

Normally, $CoQ_{10}$ is obtained from food intake, with meat being the largest source in the normal diet (48-50), as well as through endogenous synthesis. In blood, it is transported by plasma lipoproteins, primarily LDL (51), and its plasma levels are in fact considered to be an index of metabolic demand of various tissues under different physiological and pathological conditions (52). Supplementation with $CoQ_{10}$ has been shown to lead primarily to increased plasma levels, which may account for most of the reported beneficial effects of $CoQ_{10}$ supplementation in various instances and clinical medicine (2, 6, 7). Furthermore, Weber et al. (53) showed that supplementation with $CoQ_{10}$ not only increased plasma ubiquionol-10 level, but also lowered the plasma level of TBARS (thiobarbituric acid reactive substances), which are an index of lipid peroxidation in oxidative stress. However, sparing of plasma antioxidants, ascorbic acid and α-tocopherol was not observed.

TAC is a biochemical parameter suitable for evaluating the overall antioxidant status of serum or plasma resulting from antioxidant intake and/or production, and their consumption by the normal or increasing levels of oxidative stress. Therefore, measuring TAC can also be applied to optimize and monitor antioxidant therapy (32-36). Several methods have been developed to assess TAC of serum or plasma, because of the difficulty in measuring each antioxidant component separately and the interactions between different antioxidant components in the serum or plasma. These methods are all essentially inhibition methods and differ greatly. A free radical species is generated, there is an end point at which the presence of the radical is detected, and the antioxidant capacity of the added sample the end point value by scavenging the free radical (35, 36, 54). In our study, TAC was measured using the Trolox-equivalent antioxidant capacity assay described by Miller et al. (32) and commercialized by Randox company (TAS kit).

The increase of serum TAC observed in the present study, along with the increase of plasma $CoQ_{10}$, was expected. The increase of the latter is ascribed to exogenous, water-soluble $CoQ_{10}$ that was added into the dog food. The positive, significant ($p = 0.011$) correlation between plasma $CoQ_{10}$ and serum TAC, found 1 hour after $CoQ_{10}$ supplementation, indicated the contribution of $CoQ_{10}$ to the TAC of canine serum at this time point. Despite the tendency of plasma $CoQ_{10}$ to increase, serum TAC unexpectedly decreased significantly 2 hours after basal measurements. It then increased and remained at the higher level until the end of the measuring period. TAC values from all other sampling times were in general agreement with reported data (55-58). Measuring TAC may thus help in evaluating the physiological, environmental, and nutritional factors of the redox status (36). Since TAC provides an insight into the delicate balance in vivo between oxidants and antioxidants, its decrease could be due to the increased metabolic demand after food ingestion. However, a positive correlation close to statistical significance ($p = 0.052$) was determined four hours after $CoQ_{10}$ supplementation, when plasma $CoQ_{10}$ reached its peak concentration.

Albumin is the predominant circulating antioxidant agent and, with urate and ascorbate, makes the main contribution to the TAC of human plasma (32, 35-38). Measured with the Trolox-equivalent antioxidant capacity assay, albumin was shown to contribute 28% of the TAC of human serum (35). It acts as a free radical scavenger and as a chelator of transition metals and haem (37).

Albumin concentration remained within the normal reference range and did not change significantly during the 24-hour measuring period. Clearly, the above decrease in serum TAC was not accompanied by a decrease in albumin. However, positive significant correlations between albumin and serum TAC, determined at basal measurements and 40 minutes and 1, 4 and 6 hours later, indicate that albumin is a significant contributor to the TAC of canine serum, as is the case for human serum and plasma. These

results contrast with those of Nemec et al. (56), where no significant correlation was found between albumin and serum TAC in healthy beagle dogs, using the same method. The discrepancy could be due to the different equipment for determining TAC used in the two studies. The evidence from the performance of the TAS kit used here is that even slight changes in reaction conditions, such as temperature and run time, have marked effects on the apparent contributions of individual antioxidants, notably albumin (34, 59).

Basal biochemical measurements showed that, with the exception of alkaline phosphatase and inorganic phosphate, all biochemical parameters remained within the normal reference range (43, 44). All, except AP and Alb, differed significantly from the basal measurements during the 24-hour measuring period, with minor deviations from the normal range, which may be ascribed to the normal diurnal variations in dogs fed three times a day.

Variation of serum glucose concentration has many causes, including feeding, catecholamine release after excitement or fright, and the influence of glucocorticoid in stressed subjects. The dogs used in the present study were accustomed to the environment before the study and had been subjected to repeated venipuncture. Thus the variations are not attributable to stress (43, 60-62), but rather to feeding regime (61, 63). The diurnal urea oscillations are referable to feed intake, dietary protein supply and renal excretion (64). Variations in serum creatinine are normally related to muscle metabolism and muscle fibres in food (43, 65-67). Daily changes of electrolytes, sodium, potassium and chloride, are associated with water and food intake, excretion through skin, breathing, urine and faeces. Calcium and inorganic phosphate concentrations are influenced by renal clearance, absorption in intestine, resorption and deposition in bone, and shifting between intra and extracellular fluid compartments (43, 68, 69). These processes are under hormonal regulation that follows a diurnal pattern (70).

The mean AP values, with high standard deviations, exceeded the upper range of normal values at all sampling times on account of one dog. The slight increases in alanine aminotransferase activity observed in the present study are probably due to the liver's role in detoxification and the concomitant mild degree of hepatocyte injury (43). Albumin and all other proteins except the immunoglobulins are synthesized by the liver. These proteins are catabolized in all active tissues. Diurnal fluctuations in serum proteins are due to repartition of proteins according to physiological metabolic needs (43, 71).

Haematological parameters remained within the normal reference ranges (43, 45) in basal measurements. Though all the parameters, with the exception of LUC, changed significantly over 24-hours, there were minor, but not clinically important, deviations of MCH, MCHC, NEUT and LYMPH from normal values. Most of the changes can be ascribed to normal diurnal variations. The diurnal variations in the number of circulating blood cells are the result of multiple factors, such as the distribution between the marginal cell compartment among the tissues and organs of the body, influx from storage sites, cell proliferation, release of newly formed cells into the circulation, and the destruction and removal of damaged and old cells (43, 72-74). Blood erythrocyte concentrations are established by the relative rates of erythrocyte production, shifting of erythrocytes to and from splenic sinuses, and erythrocyte destruction. Erythrocyte production depends on the degree and duration of erythropoietin stimulus and the ability of precursor cells to respond to erythropoietin (73, 75). HGB concentration in the present study was related to the number of circulating RBC. Diurnal fluctuations in blood platelet concentration depend on platelet production, consumption and destruction, and on the shifting of platelets to and from the circulation (76). Platelet production and reactivity are affected mostly by the degree of cytokine stimulation, especially thrombopoietin, interleukin 6 and erythropoietin, and the number of responsive cells (77, 78). The variation in number of total leukocytes, neutrophils, lymphocytes and eosinophils during the sampling period is attributable to the influence of environmental factors such as light, activity, feeding and handling, but might also have followed an established diurnal pattern (79).

In conclusion, the results of the present 24-hour follow-up study have established the endogenous plasma $CoQ_{10}$ concentration and its correlation with TAC in healthy beagle dogs. The administration of a single oral dose of 30 mg of water-soluble $CoQ_{10}$ resulted in a statistically significant increase of plasma $CoQ_{10}$, with a peak in concentration four hours after administration. TAC was not observed to increase simultaneously, however at one hour after the administration of $CoQ_{10}$ there was a positive, significant ($p = 0.011$) correlation between these two parameters, indicating a contribution of $CoQ_{10}$ to the TAC of canine serum. There was also a positive cor-

relation close to significance (p = 0.052) when plasma $CoQ_{10}$ reached its peak value. Significant correlations between albumin and serum TAC, determined at basal measurements and other time points, indicate that albumin is a significant contributor to the TAC of canine serum. Selected haematological and biochemical parameters over 24-hours showed the diurnal variations normally found in dogs.

This study should be followed by a repeated-dose study in order to establish the influence of long-term administration of $CoQ_{10}$ on TAC in dogs, particularly in terms of correlation between the TAC and the time to reach the steady-state concentration of $CoQ_{10}$. In addition, there is a need to establish reference values of $CoQ_{10}$ in dogs to support further clinical studies on $CoQ_{10}$ implementation in the treatment of various diseases.

## Acknowledgement

The authors thank Professor Roger Pain for review of English.

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# 24-URNO SPREMLJANJE PLAZEMSKEGA KOENCIMA $Q_{10}$, CELOTNE ANTIOKSIDANTNE KAPACITETE IN IZBRANIH KRVNIH PARAMETROV PO ZAUŽITJU ENKRATNEGA ODMERKA VODOTOPNEGA KOENCIMA $Q_{10}$ PRI ZDRAVIH PSIH PASME BEAGLE

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**Povzetek:** Koencim Q ($CoQ_{10}$) je ena izmed najbolj obetavnih spojin v antioksidantni terapiji glede na njegove antioksidantne lastnosti in ključno vlogo pri delovanju mitohondrijev. Raziskave na ljudeh so pokazale pozitiven učinek $CoQ_{10}$ pri zdravljenju številnih bolezni. Kljub temu da je delovanje $CoQ_{10}$ pri ljudeh in živalih v osnovi zelo podobno, obstaja le omejeno število študij in eksperimentalnih podatkov o uporabi $CoQ_{10}$ pri psih. V okviru naše raziskave smo 24 ur spremljali koncentracijo plazemskega $CoQ_{10}$, celotno antioksidantno kapaciteto seruma (TAC – Total Antioxidant Capacity) in izbrane biokemijske in hematološke parametre pri zdravih psih pasme beagle po enkratnem zaužitju 30 mg vodotopnega $CoQ_{10}$. Namen raziskave je bil tudi ugotoviti morebitno povezavo med plazemskim $CoQ_{10}$ in serumskim TAC. Prav tako smo določili korelacije med TAC in albumini, ki so prevladujoči antioksidanti v človeški plazmi. Plazemska koncentracija $CoQ_{10}$ se je statistično značilno zvišala že 20 minut po dajanju $CoQ_{10}$ in dosegla vrh štiri ure po zaužitju $CoQ_{10}$. V nasprotju z našimi pričakovanji se vrednosti $CoQ_{10}$ in TAC nista istočasno zvečali, določili pa smo statistično značilno korelacijo med parametroma eno uro po zaužitju $CoQ_{10}$ ($p = 0.011$), kar kaže na prispevek $CoQ_{10}$ k celotni antioksidantni kapaciteti pasjega seruma. Štiri ure po zaužitju $CoQ_{10}$, ko je plazemska koncentracija $CoQ_{10}$ dosegla največjo vrednost, je bila korelacija blizu statistične značilnosti ($p = 0.052$). Pozitivne statistično značilne korelacije med TAC in albumini smo določili ob bazalnih meritvah, ter 40 minut, 1, 4 in 6 ur kasneje, kar kaže na pomemben prispevek albuminov k celotni antioksidantni kapaciteti pasjega seruma. Vrednosti izbranih hematoloških in biokemijskih parametrov so se v obdobju 24 ur spreminjale v skladu s pričakovanimi dnevnimi nihanji pri psih.

V nadaljevanju bi bilo potrebno raziskati vpliv dolgotrajnega dajanja $CoQ_{10}$ na TAC pri psih.

**Ključne besede:** koencim $Q_{10}$; celokupna antioksidantna kapaciteta; psi

Slov Vet Res 2009; 46 (3): 105-13
UDC 579.62:631.8:579.871:628.355

**Original Research Paper**

# INACTIVATION OF *MYCOBACTERIUM AVIUM PARATUBERCULOSIS* IN SHEEP MANURE

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**Summary:** Livestock manures and composts can be contaminated by Mycobacterium avium subsp. paratuberculosis (MAP) which can cause paratuberculosis – Johne's disease, an intestinal infection of domestic and wild ruminants. Therefore the inactivation of inoculated MAP ATCC 43015 in the compost and in the manure in storage was carried out. MAP ($2.0 \times 10^6$/g) was inoculated into the compost in three actively ventilated and isolated vessels and in a conventional manure storage pile. Herrold's Egg Yolk medium with supplements for MAP growth, followed by IS900 PCR for isolate identification was used. Direct PCR assessment of the persistence of sequence IS900 in the compost and manure material in parallel was implemented. Moisture, ash and ammonia content in the compost and manure specimens were determined and pH was measured. Salmonella enterica serovar Senftenberg (S. Senftenberg) was introduced into the compost and manure materials to demonstrate the hygienisation process.

The presence of MAP on the culture media was confirmed in the samples 16 and 24 hours after exposure in compost and manure, respectively. No MAP was isolated on the medium after 24 hours of exposure. However, using the PCR assay of compost specimens, persistence of MAP was proved in the compost samples until day 7 and in the manure in storage even after 21 days of exposure. *S. Senftenberg* S-73/98 was not present 24 hours after exposure either in compost or manure storage.

**Key words:** *Mycobacterium avium subsp. paratuberculosis; Salmonella enterica* serovar Senftenberg; microbial inactivation; bacterial DNA - analysis; composting; sheep manure

## Introduction

MAP infections can cause paratuberculosis and intestinal infections of domestic and wild ruminants and result in considerable economical losses in the livestock production (1). Feces, milk and semen are the main dissemination sources of MAP, and present an important epizootic risk (2, 3) depending on animal management, especially owing to overstocking or to group sizing of young animals (4, 5). The disease manifested in sub clinical forms even increases epidemiological risks, since MAP can be disseminated by infected animals for a long period without any specific clinical signs (6).

Received: 20 May 2009
Accepted for publication: 28 August 2009

MAP is highly resistant to physical and chemical factors owing to its special cell wall structure containing mycoside C, mycolic acid, peptidoglycans and lipopolysaccharides (7, 8). Most of the studies regarding the inactivation of MAP in complex environments, were conducted before 1985 (9). However, composting is the one of a most widely applied treatment methods used for bio waste biodegradation and hygienisation influenced by different factors which has an important role on microbiological inactivation (10, 11, 12, 13) (Watanabe et al. 1997; Böhm R 1998; Watanabe et al. 2002; Vinneras et al. 2003. Temperature, oxygen content, microorganisms competition and antagonisms, degradation of organic material, the increase of ammonium concentrations, pH and the composting time, were often selected to control and monitor the composting processes which are responsible for the effect of

proper decomposition and hygienisation processes (14, 15).

The objective of this study was to compare the persistence of inoculated MAP during two different manure treatment systems using commonly applied manure storage and composting of sheep manure. Composting is one of the most convenient and optimal manure hygienisation methods, and the application of in-vessel method enables good control of the method (16). Composting of livestock manure can be a method of choice in preventing MAP dissemination into the environment.

## Materials and methods

### *Design of the study*

Three ventilated composting vessels, each with the volume of 1m$^3$ (1m x 1m x 1m), and one experimental manure storage pile containing approximately 3 m$^3$ of material (2m × 2m × 1.5 m) were used for the study. Composting material was the mixture of sheep deep litter manure and pine bark with addition of water, so that the moisture content reaches approximately 65%. The composting mixture had been prepared fresh before experimental composting started. The compost temperature was controlled and limited up to 68 °C using radial fans which were also used to achieve aerobic composting process. (Fig. 1) The temperature of compost was controlled by PT 100 probes and by the "Visi DaQ"® computer program (Advantech, USA). The probes were placed in three vertical levels (16 cm, 50 cm and 66 cm above the vessel bottom) and inserted 30 cm deep into the material. The experimental manure storage pile, consisted of sheep deep litter manure only. The temperature was also measured by PT 100 probes at three vertical levels (25 cm, 75 cm and 125 cm above the ground), inserted 30 cm deep into the manure. (Fig. 1) The temperatures were measured every minute and recorded by data loggers (Testo 175 T3, Germany).

### *Physical-chemical analyses of compost and sheep manure*

Samples were analysed in two parallels. Moisture content was determined in the specimens as a loss upon drying at 105 °C – 110 °C after 24 h by weighing with the accuracy of 0.001 g (Exacta, Tehtnica Železniki, Slovenia).

The pH was determined in the liquid extract after the specimens had been oven dried (105 °C – 110 °C) (5 g). mixed with distilled water (25 g) and stabilized (10 minutes) using a calibrated pH meter (Iskra, Slovenia).

Ammonia was determined by titration, with 0.1 M NaOH, of 150 ml of distillate (acquired out of 10 g of the specimen, 250 ml $H_2O$ and 3 g MgO) blended with 50 ml of 0.1 M $H_2SO_4$ and metal red dye.

The ash content was determined as solid residua after the samples had been incubated at 550 °C for 30 minutes and weighed with the accuracy of 0.0001 g.

### *Preparation of the samples for exposure and sampling*

To apply the bacteria in compost and manure, we used diaphyses of bovine long bones as carriers. The carriers were filled up with approximately 5 grams of composting mixture or manure respectively and inoculated with the tested bacterial suspension (Fig. 2). Bone holes stand open, and therefore the

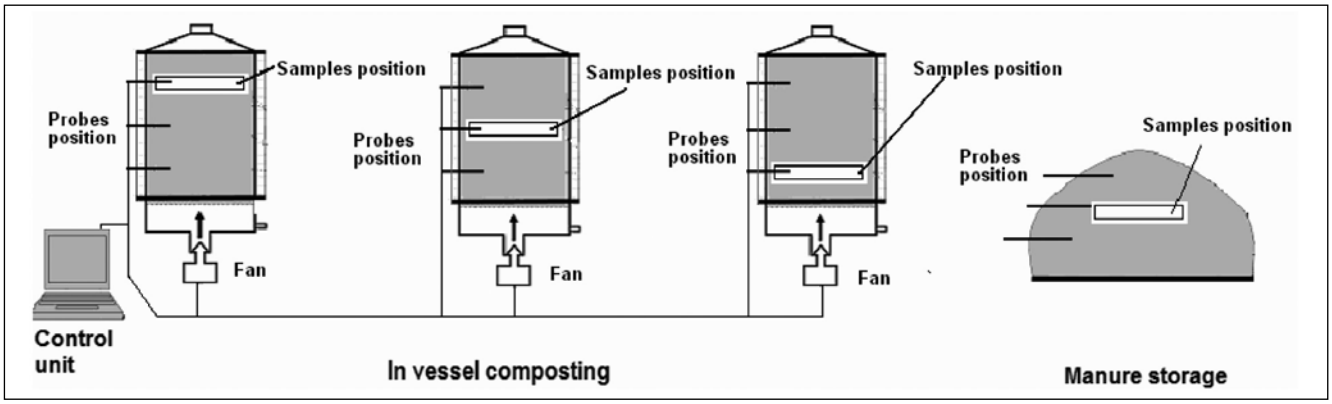


**Figure 1:** Design of the study, placing of the clusters of samples and temperature probes

material in carriers had direct contact with the surrounding material. The filled and inoculated carriers were placed in metal mesh baskets. Two holders with MAP inoculum and one carrier with *S. Senftenberg* inoculum were placed in each basket. The rest of the baskets' volume was filled up with composting material or manure respectively, and was also used for chemical analyses. Completely filled up baskets were placed in horizontal clusters (sample positions), each containing 9 mesh baskets at three different altitudes and at least 20 cm from the walls (Fig. 1). One filled up mesh basket was removed from the material at each sampling time according to the sampling scheme (Tab 1) covering the period of 21 days. So each sampling (N°) comprised six samples of MAP from the composting vessels (54 samples in 21 days) and two samples of MAP from the manure in storage (18 samples in 21 days). *S. Senftenberg* was sampled simultaneously, and comprised three samples from compost (27 samples in 21 days) and one sample from manure in storage (9 samples in 21 days). During transport to the laboratory, the mesh baskets were sealed in plastic bags and cooled below 6 °C.

Single aliquots (200 µl) inoculated into carriers contained 2.0 x $10^6$ of live MAP ATCC 43015 in suspension. The number of MAP was ascertained after 6 weeks of incubation at 37 °C by counting the colonies on Middlebrook 7H10 medium. Serial dilutions of suspension from $10^{-1}$ to $10^{-7}$ were used for inoculating (100 µl) the medium for enumeration.

The used suspension of *S. Senftenberg* (S-73/98) contained 11.7 x $10^9$/ml of live bacteria. The suspension was inoculated into the compost/manure at the ratio of 1:10. The number of live *S. Senftenberg* was determined by bacteriological method in three different media (blood agar, Rambach, xylose lysine deoxycholate agar (XLD)), and in two parallels each. Serial dilutions of the suspension from $10^{-1}$ to $10^{-7}$ were used for inoculating on the media. The colonies were counted after 24 hours of incubation.

### *Bacteriological analyses of compost/manure samples*

MAP

Two grams of the MAP inoculum was removed from each bone holder, mixed with water (50 ml), homogenized (for 30 sec.) in a stomacher (IUL, Spain), shaken for 30 minutes, and left at room temperature for 30 minutes to settle. Afterwards, 5 ml of supernatant was mixed with 25 ml of 0.9% hexadecylpyrimidum chloride (Sigma, USA) while 1 ml of supernatant was shaken again for 30 minutes and left at room temperature for 18 hours. The samples were centrifuged for 20 minutes at 1400 g (Heraeus 1.0 R, Germany). Meanwhile the supernatant was discarded, the sediment was diluted by 1500 µl of $H_2O$.

200 µl of diluted sediment was inoculated on the Herrold's Egg Yolk medium with supplements: 1. without any mycobactin; 2. with mycobactin, "3. with mycobactin and egg yolk, malachite green, penicillin and amfotericin, and on Bectom Dickinson medium. The inoculated media were incubated in 25 ml tubes in horizontal position (at 37 °C for two weeks) and vertical position (for 3 months).

### *S. Senftenberg*

The number of S. Senftenberg was determined on Rambach and XLD (Xylose lysine deoxycholate agar) medium in three parallels in up to 24 hours of exposure. Portions of 1 g each of inoculated material from the carriers was mixed, i.e. diluted with 9 ml of sterile physiological solution, and the specimen was further diluted to up to 10-5. 0.1 ml of each dilution was inoculated on the medium. After 24 hours of incubation at 37 oC, the colonies of bacteria were counted. Results with 15 - 300 colonies


Bacterial holders | Inoculation of bacteria to holders | Placing of the mash basket with holders | Order of sampling

**Figure 2:** Application of bacteria to the compost, placing of filled-up baskets and sampling order

per plate were considered as adequate for counting analysis.

The presence of *S. Senftenberg* in specimens was determined on the basis of pre- enrichment incubation of 1 g of specimens in buffered peptone medium - BPW at 37 °C for 16 – 20 hours. After pre-enrichment, aliquot of 0.1 ml of medium with colonies was transferred to selective enrichment media - Rappaport Vassiliadis Broth (RVS) and Muller-Kauffmann Tetrathionate-Novobiocin Broth (MKTTn). The specimens were incubated on RVS at 41.5 °C for up to 27 hours and on MKTTn at 37 °C also for 21 – 27 hours. After incubation, *S. Senftenberg* was determined on Rambach and XLD media by counting typical colonies after incubation at 37 °C for 21 to 27 hours.

*DNA extraction and direct molecular determination of MAP in compost material*

After extraction by QIAamp DNA Stool Mini Kit (Qiagen, Germany), DNA was amplified for IS*900* using P90 (5' GTT CGG GGC CGT CGC TTA GG 3') and P91 (5' GAG GTC GAT CGC CCA CGT GA 3') primers, respectively. After the amplification, 400-bp PCR-products were separated using electrophoresis (Power Pac 300, BioRad) on 2% agar gel stained by ethidium bromide and analyzed by scanning and a visualization system (Gel Doc 1000, BioRad, USA).

## Results

*Physico-chemical analysis*

During the first 16 hours, average hourly temperatures exceeded 50 °C in the upper and lower positions, and in the next 8 hours reached the limited maximum of 68 °C in the upper positions of composts (Fig. 3). Mean hourly temperatures (43.5 °C ± 5.2 as the 95% confidence interval of the difference, S.D. 6.8) in composts were higher than the average temperatures (23.1°C±14.2, S.D. 5.7) of the sheep manure with no correlation to outdoor temperatures in the first 24 hours (Fig. 3). In the upper positions of the composts in vessels, the temperatures over 60 °C lasted from day 2 to day 7 (148 hours), while they persisted in the range of 50 to 60 °C from day 2 to day 6 on the upper (135 hours), middle (108 hours) and lower (62 hours) positions, respectively (Fig. 4). Sheep manure was heated to more than 60 °C in the first 3 to 9 days of the test (120 hours), but after this period, the temperatures did not exceed 50°C (Fig. 2). Mean daily temperatures (47.4°C) were significantly (P=0.00) higher in sheep manure than in the composts at the middle (38.8°C±4.27, S.D. 9,3) and lower (33.2°C±4,8, S.D. 10,5) positions; however, the temperatures were lower (44.1°C±7.39, S.D. 16.3) at the upper position of compost as well (Fig. 4).

Mean hourly and daily temperatures of the composts and in the sheep manure are presented in Fig. 3 and Fig. 4.

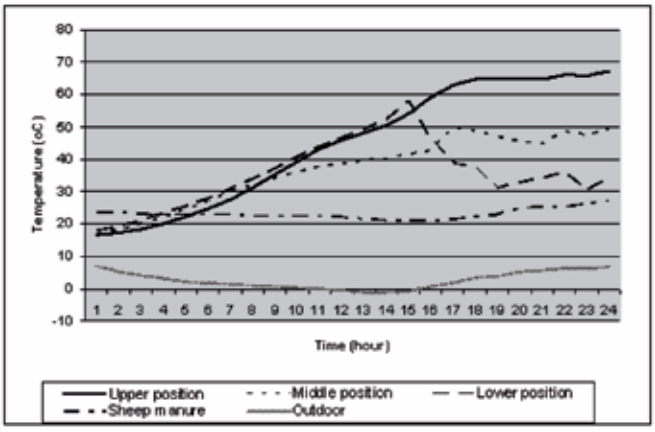


**Figure 3:** Mean hourly temperatures (°C) in composts at different sample positions and in the sheep manure in storage during the first 24 hours of the experiment

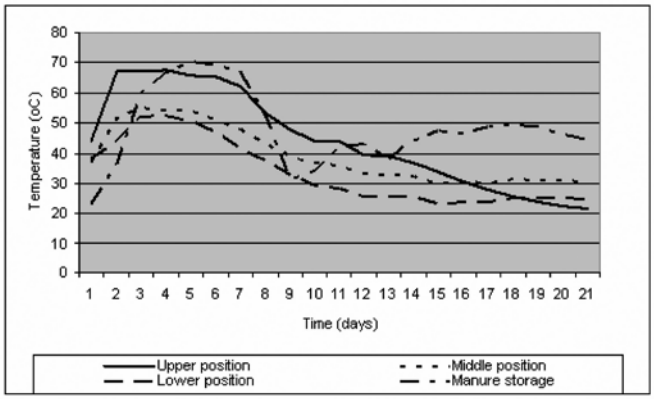


**Figure 4:** Average daily temperatures (°C) in composts at different sample positions and in the sheep manure in storage during the period of 21 days

The average moisture content of the composting mix reached approximately 65% at the beginning of the study. During the experiment, the moisture content in composts decreased (most significantly in the lower position of composts) as was not the case in the sheep manure (Tab. 1).

During the experiment, the pH values in composts varied from slightly acid to neutral (Tab. 1). The values of pH increased during the first 24 hours and persisted in nearly neutral range to the end of the study (Tab. 1). A slight trend to basification was no-

**Table 1:** Physicochemical properties of compost samples and sheep manure in storage

| Sampling interval (Nº) | Samplig day | Compost* I / Manure** II | Dry matter (g/kg) | pH | Ash (g/kg dm) | Total N (g/kg dm) | Ammonia (mg/kg) |
|---|---|---|---|---|---|---|---|
| 1 | 1 | I | 356,8±9,1 | 6,66±0,29 | 164,7±29,2 | 24,7±2,3 | 3814,8±2924,5 |
| | | II | 369,1 | 6,57 | 191,0 | 26,0 | 2961,3 |
| 2 | | I | 348,5±13,8 | 6,96±0,14 | / | / | / |
| | | II | 328,3 | 6,71 | / | / | / |
| 3 | | I | 359,3±29,4 | 6,96±0,14 | / | / | / |
| | | II | 368,8 | 7,01 | / | / | / |
| 4 | | I | 353,7±33,1 | 7,23±0,52 | / | / | / |
| | | II | 339,8 | 6,34 | / | / | / |
| 5 | 2 | I | 382,0±74,5 | 6,9±0,25 | / | / | / |
| | | II | 387,8 | 6,92 | / | / | / |
| 6 | 3 | I | 395,6±93,0 | 6,8±0,44 | / | / | / |
| | | II | 394,8 | 6,9 | / | / | / |
| 7 | 4 | I | 458,66±154,5 | 6,83±0,29 | / | / | / |
| | | II | 426,6 | 6,92 | / | / | / |
| 8 | 7 | I | 437,8±92,53 | 6,83±0,63 | 165,4±64,7 | 20,1±0,75 | 3594,3±1692,4 |
| | | II | 388,0 | 7,67 | 207,5 | 22,2 | 3605,9 |
| 9 | 21 | I | 720,7±149,4 | 6,73±0,38 | 194,5±112,0 | 22,1±6,13 | 602,9±612,2 |
| | | II | 348,5 | 7,57 | 175,0 | 31,3 | 3752,4 |

*Values are: one sample T test means resulting from biochemical analyses of composts at the upper, middle and lower position ± 95% confidence interval of the difference

** Values are: the results of biochemical analyses of the sheep manure in storage

ticed in the sheep manure on days 7 and 21, respectively (Tab. 1). Ash content increased for 18% from day 1 (164.7 g/kg dm) to the day 21 (194.5 g/kg dm) in composts; however an 8.4% decrease was noticed in the sheep manure. From the first day (24.7 g/kg dm) to day 21 (22.1 g/kg dm) the total N decreased for 10.6% in composts, but the N values increased for 20.3% in sheep manure. Ammonia content was high in raw material – sheep manure, and in compost samples (Tab. 1). Average ammonia values in compost samples recorded on day 21 (602.9 mg/kg) were 84% lower than the values recorded on day 1 (3814.8 mg/kg), while the ammonia content recorded in manure in storage even rose for 21% during the same period of time (Tab. 1).

## Microbiological analysis

Persistence of MAP determined by culture method showed that MAP was isolated in 3 samples of 24 extracted MAP inoculums from the upper and lower position of composts and in 5 of 8 samples from the sheep manure in storage during the first 24 hours after exposure to the composting process. After day 1, MAP was not isolated by culture method in any sample either in compost or in manure in storage (Tab. 2).

Using PCR-hybridization assay IS*900* by day 7, 46 of 48 samples (95.8%) were positive on MAP in all positions of samples in the compost. None of the 6 compost samples tested on day 21 were MAP positive. However, data show that 17 of 18 samples (94.4%) of the manure in storage taken during this period of 21 days were positive using IS*900* (Tab. 2).

Eight hours after exposure, the presence of *S. Senftenberg* in the compost and manure samples was between 15.8 x $10^7$ and 1.1 x $10^7$/g, while after 16 hours, *S. Senftenberg* could be isolated only in the lower position. In the period between 16 and 24 hours, the persistence of *S. Senftenberg* was not con-

**Table 2:** MAP and *S. Senftenberg* inactivation in compost and manure in storage

| *MAP* | | | | | | *S. Senftenberg* | | |
|---|---|---|---|---|---|---|---|---|
| Sampling interval (N°) | Sampling day | Sample exposition (hours) | Diagnostic method | Compost (n=6) | Manure storage (n=2) | Culture medium | Compost (n=3) | Manure storage (n=1) |
| 1 | 1 | 4 | PCR | 6 | 2 | Rambach | $4.0 \times 10^7$ | $2.9 \times 10^7$ |
| | | | Culture | 0 | 1 | XLD | $3.3 \times 10^7$ | * |
| 2 | | 8 | PCR | 6 | 2 | Rambach | $1.1 \times 10^7$ | $0.19 \times 10^7$ |
| | | | Culture | 1 | 1 | XLD | $15.8 \times 10^7$ | $13.0 \times 10^7$ |
| 3 | | 16 | PCR | 6 | 2 | Rambach | $0.2 \times 10^7$ | <1000 |
| | | | Culture | 2 | 1 | XLD | $0.6 \times 10^7$ | <1000 |
| 4 | | 24 | PCR | 6 | 2 | Rambach | <100 | <100 |
| | | | Culture | 0 | 2 | XLD | <100 | <100 |
| 5 | 2 | 48 | PCR | 6 | 2 | Rambach | 0 | 0 |
| | | | Culture | 0 | 0 | XLD | 0 | 0 |
| 6 | 3 | 72 | PCR | 6 | 2 | Rambach | 0 | 0 |
| | | | Culture | 0 | 0 | XLD | 0 | 0 |
| 7 | 4 | 96 | PCR | 5 | 2 | Rambach | 0 | 0 |
| | | | Culture | 0 | 0 | XLD | 0 | 0 |
| 8 | 7 | 168 | PCR | 5 | 2 | Rambach | 0 | 0 |
| | | | Culture | 0 | 0 | XLD | 0 | 0 |
| 9 | 21 | 504 | PCR | 0 | 1 | Rambach | 0 | 0 |
| | | | Culture | 0 | 0 | XLD | 0 | 0 |
| | | | PCR | Σ n=54 | Σ n=18 | Rambach | Σ n=27 | Σ n=9 |
| | | | Culture | Σ n=54 | Σ n=18 | XLD | Σ n=27 | Σ n=9 |

n = number of samples

firmed; the limits used for detection are stated (Tab. 2). In samples examined 24 hour after exposure the persistence of *S. Senftenberg* was excluded.

## Discussion

The method used to introduce the test microorganisms into the composting material and manure in our study followed the intention of the Opinion (17) which describes typical procedures for exposing test organisms in composting. So a typical exposure method includes contamination of raw material by the tested microorganisms and placing of the contaminated material into a carrier (*e.g.,* textile sack) protected from mechanical destruction by a perforated metal basket and recovered at the end of the process. In our study, textile sacks were replaced by natural carriers, which stayed stable during exposure to composting and provided contact with the rest of the compost/manure environment.

The purpose of the *S. Senftenberg* inoculation was a MAP independent assessment of hygienisation process of the tested materials. Bacterial species involved in biodegradation, which are more active in temperatures ranging from approx. 15°- 40°C, are termed mesophilic bacteria. We failed to define any of the mesophiles in our study, however we believe in their significant influence on the laboratory MAP diagnostics from the first samples.

In the experimental composting vessels, the MAP ATCC 43015 test microorganism was exposed to rapidly growing temperatures, high ammonia concentrations and to intensive bacterial activity. In the present study, suitable conditions for optimal microbial biodegradability processes in experimental composting mixtures were achieved. Appropri-

ate homogenization, optimization of the composition and aeration of the composting material are responsible for favourable conditions for advantageous composting (18). Temperature rise of the material during the composting process is a result of microbial oxidative degradation of organic matter, and indicates the intensity of the process outcome (19). Temperatures in composts increase very rapidly (over 50 °C in 14 hours) since the sheep manure, as a part of composting mix, originates directly from the paddock's deep litter, where biodegradation has already started.

In the laboratory diagnostics, MAP cultures on the medium can be overgrown by the fast-growing mesophile microorganisms (20, 21, 22, 23). Owing to that, we believe that the initial composting conditions during the first four hours mainly influenced the possibility of the MAP culture diagnostics in our study. The temperatures of the compost and manure did not exceed 20 - 30 °C, and therefore mesophile microorganisms survived in the samples from that time. In laboratory tests mesophiles could thus overgrow and restrict MAP growth on the culture medium, even though MAP was present in the sample (24, 10, 25).

Temperature-sensitive mesophile microorganisms were reduced with the rising of temperature over the next 16 hours, thus this situation improved the possibilities for MAP isolation until the critical temperatures reached lethal values for MAP. Therefore, only 3 samples of MAP out of 18 were positive on the medium in the period of 16 hours after the composting started and utill the compost temperatures exceeded 50 °C. After the first 24 hours of exposure no MAP was isolated on the medium in compost samples, although the temperatures did not exceed 50 °C utill the end of the study. It is obvious that in our study the starting temperatures in composts were MAP lethal. Similar results to ours were obtained by the study of Fiesinger and Harrison (26). They did not find any viable MAP in the naturally MAP contaminated substrate after the first 24 hours of composting. Olsen et al. reported that MAP survived for 21 days in mesophile conditions as compared to only few hours in termophile conditions (27). After 16 hours, positive *S. Senftenberg* isolation was still determined only at the lower compost position where temperatures fell below 50°C, while no positive samples were determined after 24 hours, above all owing to the *S. Senftenberg* lethal temperatures of the compost reaching up to 55 °C (25, 10).

Although sheep manure from paddock was transported by front loader and loaded in a heap –this manipulation stimulates oxygenation and consequently biodegradation – the process resulted in temperature increase, but not earlier than on day 3. Slow temperature changes in experimental sheep manure in storage indicate weak mesophile bacterial activity in the first 24 hours from the start of the study, and for this reason the persistence of MAP was proven on the media in almost on half of samples during this period. However *S. Senftenberg* was significantly reduced in the first 16 hours after inoculation, irrespective of the moderate starting temperatures of manure (20 °C - 30 °C). Other influencing factors for *S. Senftenberg* inactivation can be the competitive and antagonistic growth of other microorganisms' species, organic matter decomposition, ammonia concentration, pH, and the exposition time (15).

Regarding MAP determination, previous research has demonstrated that molecular methods have better sensitivity than the classical medium cultivation (22). Blocking of persistent inhibitors and optimization of DNA extraction can contribute to considerably better sensitivity of tests used (28,29). However, molecular methods did not enable us to prove viable MAP. In our study, negative MAP results determined by culture methods indicate the destruction or strong reduction of MAP in composts, but most of samples taken up to day 7 still contained intact sequence IS*900*. Data show that untill day 21 almost all samples of sheep manure were MAP positive using IS*900*. At this point of the study the question of DNA persistence in compost and manure, after the destruction of MAP cells still remains unresolved, owing to the fact that only limited data about DNA destruction during composting are available in the literature. Previous studies (30) confirmed the persistence of DNA in compost even six days after cell destruction.

At the start of our experiment, composts had relatively high average ammonia content compared to day 21 when ammonia content was more than 84% lower. This fall could be the result of active ventilation, which was not the case with manure storage process (31), where ammonia even increased by 21%. The role of ammonia influence on MAP in the compost is not clear as yet. Katayama et al. described negative influences of 3% of ammonium concentration on MAP destruction in hay, and greater influence on MAP persistence in materials with low humidity content (32). However, in our study we found

high values of ammonia in the composts and in manure (2.9 – 3.8 g/kg of dry matter), which can be an important factor in MAP inactivation.

## Conclusions

We believe that manure composting can eradicate or at least drastically reduce the number of pathogenic microorganisms, and therefore it can be used as a preventive measure against spreading of MAP into the environment. The impact of particular hygienisation factors during composting on MAP still is not clear. Although in the present study temperatures in manure were higher than in compost, DNA material of MAP in manure did not decompose even to the 21st day of experiment, while DNA were not determinate in composts to that time. At present, the factors influencing bacterial DNA degradation owing to the composting processes are poorly known and require additional scientific explanation.

The main advantages of the in-vessel composting include a more efficient composting process, and may also ensure conditions requested for suitable hygienisation of the organic material. The composting procedure in vessels can provide for a rapid decomposition process regardless of external ambient conditions. Therefore, composting in vessels can be thermophilic, thus being most convenient for compost hygienisation, and recommended for the treatment of manures intended for pathogen-sensitive environments such as those for vegetable production, residential gardening, or application to rapidly draining fields (33).

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## INAKTIVACIJA BAKTERIJ VRSTE *MYCOBACTERIUM AVIUM PARATUBERCULOSIS* V OVČJEM GNOJU

I. Gobec, M. Ocepek, M. Pogačnik, M. Dobeic

**Povzetek:** *Mycobacterium avium ssp. paratuberculosis* (MAP) povzroča paratuberkulozo – Johnovo bolezen, črevesno nalezljivo bolezen pri domačih in divjih prežvekovalcih. V raziskavi smo uporabili laboratorijski sev bakterije MAP ATCC 43015. Bakterije MAP smo vnesli v kompostno mešanico v kocentraciji 2.0 x $10^6$/g v nosilcih, in sicer v tri aktivno zračene in izolirane kompostne sode in v kup ovčjega gnoja. Preživetje bakterij MAP med kompostiranjem smo ugotavljali na Heroldovem gojišču z dodatki in z molekularno metodo PCR in insercijsko sekvenco - IS900, ki je služila za določitev izolatov. V kompostnem materialu smo sekvenco IS900 določali tudi neposredno. Med kompostiranjem smo v kompostnem materialu ugotavljali temperaturo, pH vsebnost vlage, dušika, amonijaka in pepela. *S. senftenberg* je bila vnesena v kompostni material in gnoj za prikaz higienizacijskih procesov. Bakterije MAP smo iz kompostnega materiala izolirali še 16 ur po pričetku kompostiranja, ne pa tudi po 24 urah. V kompostih smo z direktno metodo PCR ugotovili prisotnost MAP IS900 v vzorcih, odvzetih od začetka do 7. dneva raziskave, v nasprotju z gnojem, kjer smo ugotovili sekvence v vzorcih, odvzetih tudi po 21. dnevu. Vzorci *S. senftenberg* S-73/98 v kompostnem materialu in ovčjem gnoju niso bili pozitivni po 24 urah.

**Ključne besede:** *Mycobacterium avium subsp. paratuberculosis; Salmonella enterica* serovar Senftenberg; mikrobna inaktivacija; bakterijska DNK - analize; kompostiranje; ovčji gnoj

Slov Vet Res 2009; 46 (3): 115-21
UDC 636.7.09:616.453-006.55

**Original Research Paper**

# THE OCCURRENCE OF CORTICOTROPHINOMA IN CROSS-BREED AND BREED DOGS

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**Summary:** The aim of this study was to evaluate the presentation of corticotrophinoma in regard to its size and ACTH production in cross-breed and pure breed dogs. A retrospective study based on clinical histories from 2003-2008 was carried out on dogs with confirmed Pituitary Dependent Hypercortisolism (PDH) (n=100). Evaluation of the pituitary was performed by nuclear magnetic resonance imaging (NMRI) and tumours were classified according to their projection with respect to the sella turcica as intrasellar (IS) or extrasellar (ES). 26% (26/100) of the evaluated patients were cross-breeds and 74% (74/100) were dogs of different pure breeds. Both ACTH plasma concentration and urinary cortisol/creatinine ratio did not show significant differences between pure breed and cross-breed dogs. With regard to the frequency of presentation of IS and ES adenomas, no significant differences were observed neither between cross-breed and pure breed dogs nor between the different breeds studied. Thus, it can be concluded that there is no breed predilection for the presentation of IS or ES adenomas in dogs with PDH.

**Key words:** pituitary dependent hypercortisolism; corticotrophinoma; pituitary; Cushing's disease

## Introduction

Pituitary Dependent Hypercortisolism (PDH) is caused by the ACTH producing pituitary adenoma or corticotrophinoma. Its aetiology is being studied and two theories were proposed: (1, 2) the monoclonal theory that suggests a mutation of the corticotroph cell as the mechanism for tumour development and (3, 4, 5) the hypothalamic theory based on overstimulation of the corticotroph that would lead to hyperplasia and afterward, mutation of some of these hyperplasic cells developing the adenoma. With respect to the hypothalamic theory, it has been proposed that the hypothalamus would exert an overstimulation on the corticotroph area by greater secretion of CRH and AVP (3, 5). Also, defects in the hypothalamic glucocorticoid receptor could lead to a greater stimulation of the corticotroph cells due to a lower inhibitory action of cortisol (6, 7). On the other hand, dopaminergic neurodegeneration in aged individuals (8, 9, 10) or a decreased expression of the D2 dopaminergic receptor in the corticotroph cells might lead to dimnished inhibition on the corticotroph area (11, 12, 13), hence producing hyperplasia. Conseqeuently, the adenoma could develop from a somatic mutation in one of these hyperplastic cells. However, the characterization of a monoclonal state in the majority of the adenomas studied (2, 3), makes the monoclonal theory the most likely. Still, it is not clear which mutations could provoke the appearance of the tumour, and what factors are involved in its development (4). Candidate mutations would be defects in proteins that control the cell cycle (14, 15), as well as changes in the glucocorticoid and mineralocorticoid receptors and the 11-β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) (7). Considering that gene alterations could be involved in the development of the corticotrophinoma and taking into account that in breed dogs the level of homozygosis

Received: 12 July 2009
Accepted for publication: 2 September 2009

increases, a greater incidence of PDH would be expected in these animals than in cross-breeds.

The corticotrophinoma can be evaluated by nuclear magnetic resonance imaging (NMRI). In humans, NMRI would be the most precise image diagnosis method for detecting and evaluating pituitary tumours (16, 17), which can be classified according to their size to micro- and macroadenomas (5, 18). There are also studies that describe the use of NMRI in dogs with PDH with good results (19, 20). According to the adenoma projection with respect to the sella turcica, we have recently classified these adenomas as intrasellar (IS) and extrasellar (ES); thus avoiding the inappropriate evaluation of the pituitary size which varies according the size of the dog (21).

The aim of the following study was therefore to evaluate the tumour size and ACTH secretion in cross-breed and pure breed dogs.

## Materials and methods

### *Animals and tests*

A retrospective study using clinical histories from 2003-2008 was carried out on dogs with confirmed PDH (n=100) in the Endocrinology Unit of the Hospital of the School of Veterinary Sciences of the University of Buenos Aires.

Dogs were divided into two groups: cross-breeds and pure breeds, and the latter were distributed according to the breed (more than 2 dogs by breed) or as "other breeds" in case to have a maximum of 2 dogs per breed.

The following diagnostic criteria were used: presence of clinical signs associated to hypercortisolism (polydipsia–polyuria, polyphagia, pendulous abdomen, dermatologic problems, anoestrus), cortisol : creatinine ratio (C/CR) in urine greater than 70 (according to our reference values); more than 50% reduction with regard to the basal levels of C/CR after administration of 0.1 mg/kg dexamethasone every 8 hours, according to Rijnberk et al. (22) and Galac et al. (23) and increase in plasmatic ACTH, measured by immunoradiometric assay (RIA) using a commercial kit (ACTH Alpco immunoassays, Alpco Diagnostics, Salem, USA). The ACTH intra-assay and inter-assay coefficients of variation were 3.1% and 5.8% respectively. Urine cortisol was measured by RIA, using a commercial kit (DPC Corporation, San Diego, California, USA) and its intra-assay and inter-assay coefficients of variation were 5% and 8% respectively. Urine creatinine was measured by Metrolab Autoanalizer Merck, Germany, according to the manufacturer's instructions. Presence of the adenoma was confirmed using NMRI.

### *Diagnostic imaging*

To perform the study dogs underwent general anaesthesia. Evaluation of the pituitary was carried out using NMRI on sagital, axial and coronal sections, with slices every 2 mm, with gadolinium to contrast (General Electric 1Tesla). Tumours were classified according to their projection in IS or ES as it has been previously described (21).

### *Statistical analysis*

Comparison of plasma ACTH concentration, C/CR (both basal and post dexamethasone) between breeds and cross-breed dogs was performed by Mann-Whitney's test and its values are expressed as median and range. Comparison of the adenomas IS or ES between breed and cross-breed dogs were carried out by Chi Square's test followed by Exact Fisher's test. For comparison of IS or ES projection between different breed dogs Chi Square's test was used. $P<0.05$ was considered significant.

### *Ethical approval*

The study was approved by the Ethics Committee of the Faculty of Veterinary Sciences of the University of Buenos Aires and by the Secretaría de Ciencia y Técnica (Secretariat of Science and Techniques) of the University of Buenos Aires (UBACyT; V006 project) in fulfilment of the national laws on experiments with animals.

## Results

From the total number of patients evaluated, 26% (26/100) were cross-breed and 74% (74/100) were dogs of different breeds, with Poodles being predominant (Tab. 1). Average time of presentation of the first clinical signs noticed by the owners previous to PDH confirmation was 4.1 months (range of 1 year to 1 month before PDH was diagnosed). ACTH plasma concentration did not show significant differences between breed and cross-breed dogs (16.08 pmol/L [3.9-132] and 14.03 pmol/L [1.8-48], respectively) (Fig. 1). C/CR did not show significant differences between breed and cross-breed dogs, either

basal (cross-breed dogs: 210 [83-317]; breed dogs: 153 [66-756]) and post dexamethasone (cross-breed dogs: 95 [35-180]; breed dogs: 70 [30-147]). Inhibition after dexamethasone treatement did not occur in 20% (20/100) of the total dogs, showing similar proportions in both cross-breed (19.2%; 5/26) and breed dogs (20.3%; 15/74).

**Table 1:** Corticotrophinoma presentation according to NMRI in cross-breed dogs and different breeds

| Breed | Number of cases | IS | ES |
|---|---|---|---|
| Cross-breed | 26 | 7 | 19 |
| Poodle | 12 | 8 | 4 |
| Beagle | 8 | 1 | 7 |
| Schnauzer mini | 8 | 2 | 6 |
| Daschund | 8 | 4 | 4 |
| S. Husky | 5 | 3 | 2 |
| Cocker | 3 | 1 | 2 |
| G.Shepherd | 4 | 3 | 1 |
| Other breeds | 26 | 9 | 17 |

Other breeds: Shit-zu, Breton, Pitbull, Foxterrier, Boxer, Airedale Terrier, Welsh Terrier, Yorkshire Terrier, Bichon Frise, Belgian Sheepdog, Labrador, Bullterrier, Akita Malamut, Maltese, Pekinese, Pincher, Scottish Terrier, Irish Setter, Whippet, Bobtail.
S. Husky: Siberian Husky; G. Shephered: German Shepherd Dog.
IS: intrasellar, ES: extraselar

Regarding tumour morphology, a greater number of ES adenomas were observed in cross-breeds, Beagles, Schnauzer mini, Cocker Spaniel and in the "other breeds" group. In Poodles and German Shepherds a greater number of IS adenomas were observed, whereas in Siberian Husky and Daschund, presentation was similar for both types (Tab. 1 and Fig. 2). Dogs that did not show inhibition to dexamethasone presented mostly ES adenomas (16/20); and no significant differences were found between cross-breed and breed dogs, holding the same proportion than in total dogs (ES: cross-breed dogs 80%, 4/5; breed dogs 80% 12/15; IS: 20% in both cross-breed and breed dogs)

Comparing frequency of presentation of ES and IS adenomas between breeds and cross-breeds, no significant differences were observed neither between groups nor between the different breeds studied.

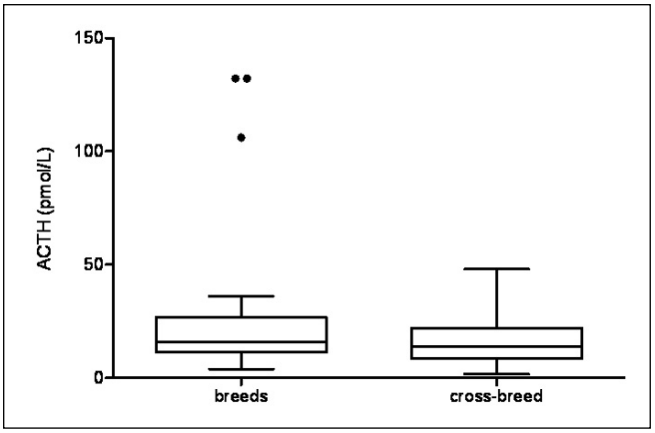


**Figure 1:** Plasma ACTH concentration (pmol/L) in pedigree and cross-breed dogs. Full circles represent 3 outlier values corresponding to dogs with ES adenomas (G. Shepherd, Schnauzer and Siberian husky)

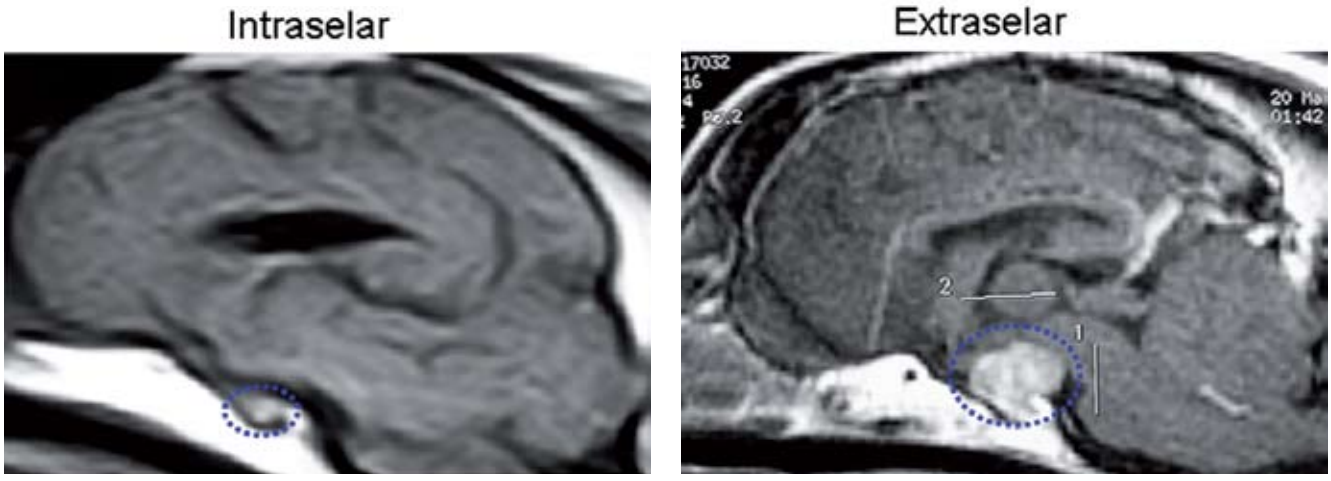


**Figure 2:** Nuclear magnetic resonance imaging (NMRI) on sagital slice. Examples of intrasellar (left) and extrasellar (right) projection of the adenoma in 2 dogs. The adenoma is encircled with dotted line. Intrasellar tumour does not exceed the upper limit of the sella turcica and shows an increase of the paramagnetic signal. In contrast, the extrasellar tumour is expanding to the hypothalamic area and is showing irregular appearance

## Discussion

Variations according to the type of corticotrophinoma regarding hormone production, size and resistance to corticoid inhibition as well as sex and age of presentation have been established (18, 21, 24). In this study we did not find significant differences in adenoma presentation (IS or ES), ACTH plasma concentration and C/CR between cross-breed and pure breed dogs, nor between different dog breeds.

Although a higher prevalence of PDH has been described in Poodles, Daschund and Terriers (25), this could be caused by temporal preferences for certain breed in different countries. In South America, cross-breed dogs are usually preferred, while different breed preference varies with trends and in different time periods. These preferences might determine the higher prevalence of cross-breed dogs with PDH in our country (Argentina), comparing this group with the different groups of each pure breed. However, according to our study, no significant differences were found between cross-breed and pure breed dogs. While the higher level of homozygosis in breed dogs could lead to the expectations that these animals could have a greater tendency for developing corticotrophinomas; based on our results, breed is not a risk factor for developing PDH. Other factors, independent of the genetic background, are most likely involved.

We have noticed that cross-breed dogs and certain breeds show a different kind of presentation with regard to the adenoma projection (ES or IS). It is possible that specific molecular defects more often occur in certain breeds. The fact that the time elapsed since the appearance of the first clinical signs and the diagnosis is not very long suggests that different sizes of corticotrophinomas are caused by different molecular alterations, as it has been described by many authors (1, 4, 18, 26). Thus, corticotrophinomas would not grow indefinitely; they would grow until reaching a self-limiting size. However, some of these tumours, usually macroadenomas, show a different behaviour: they not only present a sustained growth but they are also resistant to endogenous and exogenous glucocorticoid inhibition (18, 24). These findings are consistent with our study, where 80% of dogs that showed lack of inhibition to dexamethasone, presented ES adenomas, independently of their breed. Nevertheless, it must be considered that 20% of the adenomas, not inhibited by dexamethasone, are IS. It might be possible that these tumours grow over time, especially if steroideogenic inhibitors are used as medical treatment, as it has been described by Teshima et al (27). From these studies follows the importance of combining diagnostic tests with NMRI and the use of a specific treatment such as ACTH synthesis/tumour's growth inhibitors or surgical removal of the adenoma (28, 29, 30).

More studies are necessary to clarify which factors can provoke gene alterations (mutations, loss of heterozygosity, mRNA translation or transcription defects, etc.) or affect the hypothalamus-pituitary-adrenal axis regulation, leading to the development of the corticotrophinoma. The study by Teshima et al (7) suggested the GR defect is a major contribution to the tumour development, although this has not been yet conclusive.

Cell cycle proteins, particularly Rb and p27, have been reported to be associated with aggressive corticotrophinomas that develop from the intermediate lobe in mice (14, 31).

In summary, according to our study there is no genetic predisposition to develop neither PDH nor a particular type of adenoma (IS or ES); and pure breed dogs are not more susceptible than cross-breed dogs to develop the corticotrophinoma.

## Acknowledgments

This project received funding from UBACyT, University of Buenos Aires (V006).

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## POJAVNOST KORTIKOTROPNIH TUMORJEV PRI MEŠANCIH IN ČISTOKRVNIH PSIH

M. F. Gallelli, D. D. Miceli, M. F. Cabrera Blatter, M. M. Brañas, V. A. Castillo

Povzetek: Namen naše raziskave je bil proučiti pogostnost kortikotropnih tumorjev hipofize glede na nijihovo velikost in proizvodnjo kortikoliberina pri psih mešancih in čistokrvnih psih. Retrospektivna raziskava je zajela 100 psov, ki so imeli v letih 2003 do 2008 potrjeno diagnozo od hipofize odvisnega hiperkortizolizma. Hipofize so bile pregledane s pomočjo jedrske magnetne resonance, tumorje pa smo razdelili glede na njihovo velikost v tiste, ki so bili samo znotraj turškega sedal in na tumorje, ki so segali preko njegovih robov. Šestindvajset odstotkov preiskanih psov je bilo mešancev, 74 % pa je bilo čistokrvnih psov različnih pasem. Raven kortikoliberina v krvni plazmi in razmerje med kortizolom in kreatininom v seču se ni razlikovalo med mešanci in čistokrvnimi psi. Prav tako ni bilo razlike med mešanci in čistokrvnimi psi v pogostnosti pojavljanja tumorjev, omejenih znotraj turškega sedla ali tumorjev, razširjenih izven turškega sedla, prav tako pa nismo opazili nobenih razlik med pasmami čistokrvnih psov. Raziskava tako kaže, da pasma psov ne vpliva na pojavnost in hitrost rasti kortikotropnih tumorjev pri psih.

**Ključne besede:** od hipofize odvisen hiperkortizolizem; kortikotropni tumor; hipofiza; Cushingova bolezen

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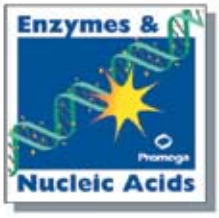






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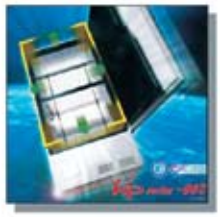

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### Examples of references

**Book:** Hawkins JD. Gene structure and expression. Cambridge: University Press, 1991: 16.

**Chapterorar ticle in a book:** Baldessarini RJ. Dopamine receptors and clinical medicine. In: Neve KA, Neve RL, eds. The dopamine receptors. Totowa: Human Press, 1996: 475-98.

**Article in a journal or newspaper:** Fuji J, Otsu K, Zorzato F, et al. Identification of mutation in porcine ryanodine receptor asociated with malignant hyperthermia. Science 1991; 253: 448-51.

**Article in proceedings of a meeting or symposium:** Schnoebelen CS, Louveau I, Bonneau M. Developmental pattern of GH receptor in pig skeletal muscle. In: the 6th Zavrnik memorial meeting. Lipica: Veterinary Faculty 1995: 83-6.

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### Načini citiranja

**Knjiga:** Hawkins JD. Gene structure and expression. Cambridge: University Press, 1991: 16.

**Poglavje ali prispevek v knjigi:** Baldessarini RJ. Dopamine receptors and clinical medicine. In: Neve KA, Neve RL, eds. The dopamine receptors. Totowa: Human Press, 1996: 475-98.

**Članek iz revije ali časopisa:** Fuji J, Otsu K, Zorzato F, et al. Identification of mutation in porcine ryanodine receptor asociated with malignant hyperthermia. Science 1991; 253: 448-51.

**Članek iz zbornika referatov:** Schnoebelen CS, Louveau I, Bonneau M. Developmental pattern of GH receptor in pig skeletal muscle. In: the 6th Zavrnik memorial meeting. Lipica: Veterinary Faculty 1995: 83-6.

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