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CHANGES IN BEHAVIOURAL AND PHYSIOLOGICAL PARAMETERS ASSOCIATED WITH *TAENIA PISIFORMIS* INFECTION IN RABBITS (*ORYCTOLAGUS CUNICULUS*) THAT MAY IMPROVE EARLY DETECTION OF SICK RABBITS

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Abstract: The purpose of this experiment was to describe early behavioural responses to illness in rabbits and their relation with physiological changes to improve early detection of infection by *Taenia pisiformis* in rabbits. Twenty adult female New Zealand rabbits were randomly allocated to 2 groups to determine whether changes in behaviour and some physiological parameters can be induced in rabbits after a *T. pisiformis* infection. Infected animals were orally inoculated with 3000 eggs of *T. pisiformis*, while controls received only saline solution. Behavioural activity was recorded daily from 19:00 to 21:00 h starting 2 d before infection. Mate choice and rank status were assessed, and blood samples were collected at −2, 7, 14 and 25 d post infection (dpi) for hematological and hepatic function determinations. All animals were observed for clinical signs every other day from the beginning of the experiment and euthanised 25 dpi after last sampling. Infected animals spent more (P<0.01) time lying stretched (3.78±1.77 *vs*. 0.77±0.03%) and less (P<0.01) time grooming (1.95±1.31 *vs*. 2.58±0.10%) and at the watering trough (1.20±1.13 *vs*. 3.35±0.02%) than controls. These differences were noticeable 6 dpi and remained until the end of the experiment. No changes (P>0.05) were observed in the time spent at the feeder, rank status or mate choice. Leukocyte and lymphocyte concentrations increased (P<0.05), while heterophil counts decreased in infected rabbits as the experiment progressed. Furthermore, infected animals had larger concentrations of alkaline phosphatase as soon as 7 dpi. No clinical signs of the disease were detected. Necropsy findings corroborated hepatic lesions and presence of the parasite in all infected animals. It was concluded that an infection with 3000 eggs of *T. pisiformis* induced changes in behavioural and physiological parameters that may improve early detection of sick rabbits.

Key Words: *Taenia pisiformis*, behaviour, parasite infection, rabbits, hepatic function.

INTRODUCTION

The domestic rabbit (*Oryctolagus cunuiculus*) is susceptible to infections of *T. pisiformis*. The cycle of this parasite starts when the rabbit ingest the eggs of this worm, present in the faeces of carnivores such as dogs, cats or foxes. These eggs travel through the digestive tract of the intermediate host reservoir. The eggs hatch and larvae migrate toward the liver, mesentery, peritoneum and lungs. The cycle is completed when carnivorous definitive hosts eat the parasitised rabbits, developing the adult state of the parasite in their intestine, allowing sexual reproduction of the latter (Flatt and Moses, 1975).

Once sick, host behaviours are directed to the recovery of the animal. Sick animals behave very differently from those that are not (Orihuela and Vázquez-Prats, 2008); they seem depressed, lethargic, and have

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scarce or null appetite, isolating themselves from the normal social activities of the group, a syndrome that has been defined as sickness behaviours (Hart, 1988). However, limited data on behavioural and physiological welfare indicators are available for rabbits (Verga, 2005; Trocino and Xiccato, 2006).

Clinical signs may also appear, contributing to the diagnosis of the disease. Symptoms such as diarrhoea, abdominal distension and pain, blood or mucus in faeces are indicative of parasitic diseases. However, diagnosis can only be confirmed with the physical finding of the parasites.

Some researchers have established that changes in behaviour may appear before the clinical signs, improving early detection of illness (Flower and Weary, 2006; Pastell and Kujala, 2007; Svensson and Jensen, 2007). For example, 10 wk after an experimental infection, even though there were no signs of gastrointestinal parasitism, calves that were not treated with an ivermectin bolus grazed on average 105 min less per day, consuming 0.78 kg less dry matter when compared to treated animals (Forbes *et al*., 2000). Feeding data from automated milk feeders have been used to help detect illness in milk-fed calves (Svensson and Jensen, 2007). More specifically, monitoring changes in feeding behaviour have helped to identify cows at high risk of metritis (Huzzey *et al*., 2007) and even changes of dry matter consumption on the day of calving have also been used to assess cows' lameness (Proudfoot *et al*., 2007).

In general, a better knowledge of animal behaviour related to illness will help to understand the causes of the illness and might improve its early determination (Rushen *et al*., 2007). With this in mind, the aim of the present experiment was to describe early behavioural responses to illness, and their relation with physiological changes, to help improve timely detection of infection by *T. pisiformis* in rabbits.

T. pisiformis was selected for a number of reasons. In addition to producing illness in intensive production species, host behaviour alterations could explain selective pressure which has enhanced transmission of the parasite from its intermediate host reservoir to its definitive host. Due to the characteristics of the biological cycle of *T. pisiformis*, this parasite has been used as a model for the study of other important zoonosis relevant to human health. For example, *T. pisiformis* has the advantage of being innocuous to humans while *T. solium* represents a main human health problem in developing countries. In addition, *T. pisiformis* and *T. solium* share several oncospheral antigens and the metacestode antigen of the later contains epitopes cross reactive with rabbit anti-sera to adult and oncospheral stages of the parasite (García-Allan *et al*., 1996).

MATERIALS AND METHODS

Animals

Twenty adult New Zealand female rabbits between 180-185 d old, with an average body weight of 2.67±0.09 kg, were used. Animals were housed in individual cages (60×90×40 cm), elevated 70 cm from concrete floor, and received a commercial concentrate (16% crude protein) and water *ad libitum* during the trial. All animals were kept under natural temperature (19±3°C) and light (11 h light; 13 h dark) conditions.

At the beginning of the experiment, no rabbits had any history of parasitic infection or signs of disease.

Experimental procedure

Animals were allocated randomly to 2 groups. In the infected group, 10 rabbits were orally inoculated with 3000 eggs of *T. pisiformis*, while the rest of the animals were not infected (controls).

General health status of the animals was checked by a veterinarian every other day until the end of the study (25 dpi).

Infection with T. pisiformis

Adult metacestodes of *T. pisiformis* were obtained from the guts of adult dogs euthanised in the Canine Control Centre at Tlahuac County in Mexico City. Each intestine was carefully inspected after a longitudinal cut in search for the Taenia in the intestinal lumen. Parasites were identified, washed and stored at 4°C according to the methodology proposed by Flatt and Campbell (1974).

After refrigeration, egg viability was 80% (technique described (Wang *et al*., 1997), and a total of 3000 eggs were counted using a Newbauer camera. This was the infecting dose used per rabbit (Flatt and Moses, 1975; Worley, 1974).

A flexible sterile oral probe was used to inoculate each rabbit with the parasite. The same procedure was repeated in the control animals, inoculating only phosphate buffered saline (PBS). Before this practice, animals were tranquilised with 35/5 mg/kg im ketamine/xylazyne, respectively (AVMA, 2001).

Time budgets

All animals were observed continuously from 19:00 to 21:00 h using a time-lapse video recorder every day until the end of the study (25 dpi,) starting 2 d before infection from September to October, 2008. Cameras had a night recording option (allowing recording with minimum light), as sunset occurred at 19:12 the day of infection, and at 19:24, 25 d later.

The length of the observation period was established on the basis of behavioural studies by Gonzalez *et al*., 1990 and 1993, while the moment in time was set based on Gunn and Morton (1995), who found that behaviours like lying, grooming and eating occurred more during the dark, and the periods of greatest social activity take place during evening and night.

For each rabbit the total time (within the 2 hs of observation) that a particular behaviour occurred was converted into a percentage for each behaviour (e.g. for one rabbit, the mean grooming time for the 25 d was 2.4 min). This rabbit groomed for 2% (2.4×100/120) of her recording period (120 min). For each behaviour observed, the % value from individual rabbits were added together and divided by the number of animals in each treatment (n=10) to give a mean percentage and standard error value for the whole group over the 2 h observation period.

The behaviours recorded included:

a) Time spent at the feeder or watering trough (started with a rabbit putting her head in the feeder or watering trough, and ended when the animal moved away at least one step).
b) Time spent grooming. Groom was considered when the rabbit was licking or using its teeth on the coat or washing her ears with the forepaws (Gunn and Morton, 1995; Krohn *et al*., 1999) and
c) Time lying stretched (resting with body trunk on the floor, hind limbs outstretched and belly exposed (Hansen and Berthelsen, 2000).

Mating attractiveness choice test, dominance status and weight

Mating attractiveness tests were performed comparing the mating preferences of 3 sexually mature, vasectomised New Zealand males to infected and non-infected females. This rabbits had a history of high libido and weighed 3.0 kg at the beginning of the experiment.

In each test, 2 different females were introduced to each male cage; an infected and control female as mating options, in random order. Mating tests were designed to last 90 s in duration, unless ejaculation occurred (whichever occurred first).

The first approach to the female, the number of approaches toward each female, and the female mated by the male (ejaculation as described by Gonzalez, 2004) were registered.

Rank status was assessed using food as the motivational basis for competition. Food was withdrawn 5 h before each test to generate sufficient motivation. The experiment consisted of observations of pair-wise competition tests (Erhard *et al*., 2004). Animals were ranked according to competitive abilities based on counts of wins and losses of access to a feeder.

The test cage was 120×90×40 cm. In one side of the cage was a feeder (as in their home cage), which allowed access to food to only one rabbit at a time. Because one animal participated in several tests each day, animals were not allowed to eat until their last competition. Only the pairs that involved one treated and one control rabbit were considered.

Mating test choices and dominance status determination were conducted at −2, 7, 14 and 25 dpi.

Live weight was registered at noon, every day, by the use of a digital scale, until the end of the study (25 dpi) starting 2 d before infection.

Hematological and hepatic function

The site of sample collection on the ear was prepared by shaving the area and then wiping with 70% isopropyl alcohol. Once prepared, an angiocath (22-gauge, 1-in., Abbocath, Abbot Medical, Abbot Park, IL) was placed in the auricular (central) artery, according to the process described by Fuentes and Newgren (2008). Six mL blood samples were collected from both groups at −2 d (baseline), and again in days 7 and 14 dpi. Animals were tranquilised 15 min prior to blood draw with ketamine/xylazyne 35/5 mg/kg im, respectively. Cardiac blood collection was limited to the sample obtained 25 dpi from euthanised animals. Each sample was separated into 2 tubes, one containing EDTA (anticoagulant) and one without it, to perform the hematological and hepatic function analysis, respectively.

A Coulter Counter® T-540 (Coulter Electronics Inc., Florida, USA) was used to evaluate: leukocytes, heterophils, lymphocytes and basophils, while a Cobas Mira® Chemistry Analyzer (Roche Diagnostic Systems Inc., New Jersey, USA) was used for the analysis of the hepatic function as total bilirubin, alanine aminotransferase (ALT) and alkaline phosphatase (ALP) concentration (Bortolotti *et al*., 1989).

Parasitic load determination

Rabbits were euthanised by an overdose of pentobarbital (100 mg/kg) via intra-cardiac at 25 dpi. Before this practice, animals were treated with 35/5 mg/kg im ketamine/xylazyne, respectively (AVMA, 2001).

The respiratory and gastrointestinal tracts were removed for parasite examination and worm burden was assessed by visual inspection. Organs and thoracic and abdominal cavities were inspected to quantify *T. pisiformis* metacestodes (Bowman, 1999).

Statistical analysis

The Mann Whitney test was used to compare individual behaviour; the Bayesian analysis of linear dominance hierarchies (Adams, 2005) based on paired comparisons was used to compare rank status between groups; while data from mating tests were analysed to determine whether the proportion of choices deviated significantly from a 50:50 ratio within each variable.

To compare live weight, hematological and hepatic values among groups and the sequential data recorded on the same set of animals, repeated measures analysis of variance model was used:

$$Y_{ik}=\mu + \alpha_i + \beta_k + (\alpha\beta)_{ik} + E_{(ik)}$$

Where $n_1=10$; $n_2=10$; i=1, 2: effect of the ith treatment (T0 and T1); k=1-4 (sample points); μ: mean of the distribution of Y; α_i : effects of the two treatments (A); β_k : effect of time at the various sampling points in the process of repeated measurement of the subjects (B); $(\alpha\beta)_{ik}$: the interaction of A and B; $E_{(ik)}$: the

residual error (SAS, 1988). The means were compared for significance by Tukey´s test (Snedecor and Cochran, 1989) at $P<0.05$.

RESULTS

Behaviour

Infected animals spent more ($P<0.01$) time lying stretched (3.78±1.77 *vs*. 0.77± 0.03%) and less ($P<0.01$) time grooming (1.95±1.31 *vs*. 2.58±0.10%) and at the watering trough (1.20±1.13 *vs*. 3.35±0.02%) than controls (Table 1). However, no difference ($P>0.05$) was found in the time that animals spent at the feeder.

Infected animals spent less time at the drinking trough than controls and this difference was significant from 6 dpi until the end of the experiment. At 7 dpi, infected rabbits also started spending more time lying down, while the effect on grooming behaviour was observed from 8 dpi (Figure 1).

Mating tests choice, dominance status and weight

In all mating tests, the males ejaculated before the end of the 90 s period, approaching uninfected females in 58% of the tests, displaying 53% of the approaches toward these same females and mating (ejaculation) with them 55% of the times. These findings were not statistically different ($P>0.05$) from the 50% probability of success (or failure) expected at random.

All food paired tests competitions were finished before the end of the 60 s period. Rank status at all post infection sample points (7, 14 and 25 dpi) were similar ($P>0.05$) from those obtained 2 dpi. Infected females won about 50±7% of the encounters with control rabbits.

There was no difference ($P>0.05$) in live weight between treated and control rabbits at any time during the whole experiment. At 25 dpi, treated and control rabbits weighed 3.1±0.4 and 3.2±0.2 kg, respectively.

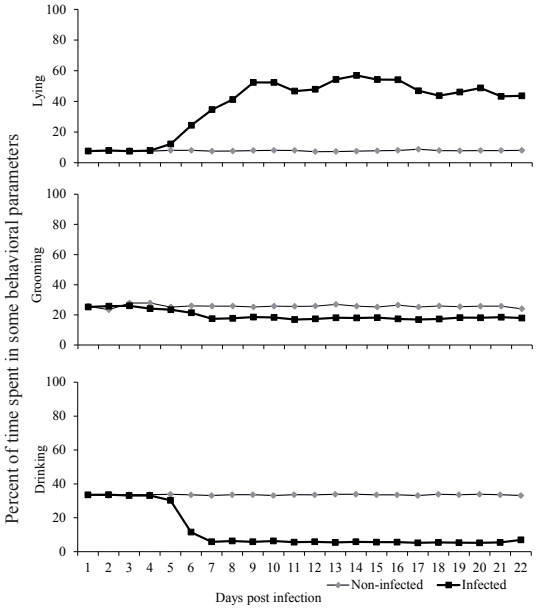

Figure 1: Dynamics in the time spent lying down, grooming and drinking in controls and treated animals from the day of infection to 25 d post infection.

Hematological and hepatic function

Infected animals showed higher ($P<0.05$) concentrations of leukocytes and lymphocytes at 25 dpi. More ($P<0.05$) heterophils were also found at 7 dpi and basophils cells were higher ($P<0.05$) at 14 and 25 dpi in comparison with controls (Table 2).

In the infected animals, total bilirubin values increased ($P<0.05$) at 25 dpi, ALT values decreased at 7 dpi ($P<0.05$), but rose to original levels ($P<0.05$) at 14 and 25 dpi. In addition, ALP concentration increased ($P<0.05$) from 7 dpi until the end of the experiment (Table 2).

Parasitic load determination

The average number of metacestodes and hepatic lesions per infected animal found during

Table 1: Percentage of total observation time (mean±standard deviation) that rabbits spent grooming, lying and at the drinking and feeding trough, when compared between infected and non-infected groups.

	Non-infected	Infected	*P*-value
Grooming	2.58±0.10	1.95±1.31	< 0.01
Lying stretched	0.77±0.03	3.78±1.77	< 0.01
At the watering trough	3.35±0.02	1.20±1.13	< 0.01
At the feeder trough	2.36±0.14	2.37±0.01	> 0.05

necropsies at 25 dpi was 2.30±1.25, and 3.70±2.66, ranging from 1 to 4 and 1 to 8, respectively. All infected animals had metacestodes of *T. pisiformis* and hepatic lesions, the latter, displayed as 2 mm white spots. However, control animals had neither hepatic lesions nor parasites.

DISCUSSION

Behaviour

In our study, feeding and grooming accounted for 5.52 and 8.29% of the time spent by non infected and infected rabbits, respectively. These parameters are below the 10% reported by Gunn and Morton (1995) and Krohn *et al*. (1999) for caged rabbits. However, this difference might be due to the more restricted observation period used during this study.

The increase in time spent lying down displayed by infected rabbits could be part of the behaviour of sick animals. The behavioural changes induced by the parasites might be based on the form of transmission involved. When the lifecycle of the parasite needs an intermediate host, as *T. pisiformis* does, then survival and reproduction of the parasite depends on predation of the infected rabbits (Klein, 2003). The decrease in the activity from the infected rabbits could also be due to general weakness (Alzaga *et al*., 2007). *T. pisiformis* metacestodes make relatively large cavities in organs and tissues of host (usually up to 1 cm of diameter). These stages demand metabolic resources from the host to survive and induce a costly immune defence with large deposition of host tissue around the lesion (Anderson *et al*., 1981). In addition, *T. pisiformis* metacestodes are located across the thoracic and abdominal organs (Anderson *et al*., 1981) and subsequently also constrain the function of affected organs and compress thoracic and abdominal muscles and bones, which could affect the running capacity of parasitised animals. It has

Table 2: Hematological and hepatic function values in infected rabbits at 2 d before infection, 7, 14 and 25 d post infection.

	Days post infection			
	−2	7	14	25
Leukocytes ($\times 10^9$/L)	5.2±0.3^{a}	5.6±0.5^{ab}	6.8±0.4^{ab}	7.3±0.5^{b}
Heterophils (%)	53.2±5.5^{b}	62.1±4.5^{b}	42.5±3.8^{ab}	33.3±3.4^{a}
Lymphocytes (%)	41.2±2.2^{a}	44.5±7.7^{a}	49.9±4.0^{ab}	57.5±4.5^{b}
Basophils (%)	0.9±0.2^{a}	0.9±0.1^{a}	2.5±0.8^{b}	2.4±0.4^{b}
Total bilirubin (µmol/L)	4.5±0.8^{ab}	2.0±0.1^{a}	1.7±0.1^{a}	5.6±0.6^{b}
Alanine aminotransferase (u/L)	31.0±2.7^{a}	10.5±1.2^{b}	24.2±1.8^{ab}	39.5±2.9^{a}
Alkaline phosphatase (u/L)	104.7±7.5^{a}	170.3±19.8^{b}	175.3±20.5^{b}	163.1±7.7^{b}

^{a,b} Different superscripts in a row refer to statistical differences (P<0.05).

been observed that larger loads of *T. pisiformis* reduce escape success from predators in free living hares (Alzaga *et al*., 2007). Other parasitic infections known to modify hosts' behaviour are *Trichinella spiralis* (Rau, 1983) and *Toxoplasma gondii* (Webster, 1994).

During the present study, infected rabbits did not reduce the time spent at the feeder. This finding is in accord with the similar body weights found at the end of the experiment in treated and control rabbits. A light load of parasites and/or a short experimental phase could be involved. Similar results were observed by Borderas *et al*. (2007) in calves, which also decreased the time spent grooming and increased inactivity while food consumption was not affected.

The degree of anorexia may be determined by species and the site of infection as well as the breed, age and resistance of the host (Sykes and Greer, 2003). Of course, the magnitude of infection is also important: infections with less than 3000 larvae of *Trichostrongylus colubriformis* per week do not reduce food consumption yet more than 30000 larvae infections do so, causing clinical diarrhoea, and death (Steel *et al*., 1980). Similarly, anorexia did not occur until 3 wk after larvae infection, coinciding with the establishment of the mature worms (Kyriazakis *et al*., 1994). *T. pisiformis* metacestodes develop in 15-30 dpi. However, hepatic lesions due to larvae stage have been reported to occur as soon as 2 dpi (Flatt and Cambell, 1974).

The lack of interest in grooming could be directly related to the increase in time spent lying (inactive). Both behaviours involve a reduction in physical activity and the consequent energy saving (Orihuela and Vázquez-Prats, 2008). However, special attention should be considered in the decrease in the time that the rabbits spent close to the drinkers. For example, Madsen and Kristensen (2005) found a decrease in drinking behaviour for up to 6 d, followed by an increase in drinking, which predicted outbreaks of diarrhoea in piglets.

Unfortunately in the present study, food and water consumption were not measured and can only be associated with the time that the animals spent at feeding and drinking troughs. However, changes in food and water consumption require further investigation

Mating tests choice, dominance status and weight

The fact that no differences were found in the mating tests is in disagreement with results from *T. crassiceps* infections, where mice males identified and avoid mating with infected females, and in some cases even displayed aggression towards them (Gourbal and Gabrion, 2004). However, regardless of the parasite species, methodological differences may contribute to these apparently contradictory results. In the Gourbal and Gabrion (2004) study, infection was not induced with eggs orally administered, but with metacestodes injected directly in the peritoneum of mice, resulting in an increase in weight and alteration of their aspect. Other possibility could be that higher parasitic loads generally induce diarrhoeas, lack of grooming and weight losses, leading to females with bad odour, unkempt fur and poor body condition, features that might be avoided by the male when selecting a sexual partner. It is important to note that these experiments were conducted in rodents, and no information is available in lagomorphs.

T. gondii and *T. cruzi* turn dominant mice into submissive mice (Arnott *et al*., 1990; Kristennson *et al*., 2002). This change in hierarchy status was not found in the present experiment. Again, perhaps increasing the length of the study and/or using larger parasite loads might have led to different results.

Hematological and hepatic function

The increase in leukocytes observed in the infected animals was gradual, starting as early as 7 dpi, a characteristic that was also observed in sheep naturally infected with *Fasciola hepatica* (Matanović *et al*., 2007).

Royo (1986) found that pigs infected orally with eggs of *T. solium*, total leukocytes and lymphocytes rose at 30 dpi, which is in agreement with the findings of the present experiment (25 dpi). This increment, even though it is not specific for parasitic diseases, could be used to drive this diagnosis.

The increase in leukocytes has been associated with acute infectious processes (Jain, 1993) that induce the liberation of cytokines which are responsible for the behaviour of sick animals (Krueger and Majde, 1994; Laye *et al*., 1994; Krueger *et al*., 2003). Even though in the present work a significant increase in lymphocytes was detected at 25 dpi, this was the result of a gradual (non significant) increase observed in samples from 7 and 14 dpi. It is possible that these small rises in lymphocytes could increase cytokine levels inducing changes in behaviours such as: time spent drinking, lying down and grooming 7 dpi. For this reason, it is suggested that cytokine concentrations should be determined in further studies at several dpi.

The increase in heterophils in the present experiment is in agreement with Pérez-Torres *et al*. (2002), who reported that a local inflammatory response is characterised by a significant increase in heterophils. In addition, basophils participate in the immediate hypersensitivity reaction, producing histamine. Melman (1987) found that the basophiles rose in parasitic diseases involving *Ancylostoma caninum*. Similar results were found in the present experiment at 14 and 25 dpi.

The increase observed in total bilirubin may indicate post hepatic jaundice or obstruction. In addition, a raise in ALT concentration is generally associated with hepatocyte necrosis (Presidente *et al*., 1975; Kraft and Schillinger, 1989). However, the importance of the latter in the diagnosis of parasitic diseases has not been established (Vengust *et al*., 2003).

An increment in ALP was observed 7 dpi. This rise suggests an obstruction in the hepatic tissue due to the presence of the parasite. An increase in this enzyme has also been reported in sheep (Matanović *et al*., 2007) and deer (Vengust *et al*., 2003) infected with *F. hepatica*. For this reason, this enzyme has been proposed as a reference in parasitic diseases characterised with hepatic migration (Conboy and Stromberg, 1991).

Parasitic load determination

The fact that no metacestodes or hepatic lesions were found during necropsies in the controls, while all infected animals showed these situations, confirms the success of the infection process and the origin of the changes recorded.

In summary, an infection with 3000 eggs of *T. pisiformis* induced changes in the behaviour of rabbits starting 6 dpi, reducing the time animals were observed at drinkers and spent grooming, while increasing the time spent lying down. There was also a simultaneous increase in leukocytes and alkaline phosphatase associated with the behavioural changes. These physiological reactions are related to the production of cytokine pro-inflammatory responses and hepatitis due to the parasite's presence in the liver.

This study is a novel description of behavioural alterations of domestic rabbits induced by the experimental infection of *T. pisiformis*.

CONCLUSIONS

An infection with 3000 eggs of *T. pisiformis* induced changes in behavioural and physiological parameters that may improve early detection of sick rabbits.

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