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Impact of *Theileria equi* Infection on Arabian Stallion Fertility: Serological and Hormonal Perspectives

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ABSTRACT

Theileria equi, a protozoal parasite transmitted by ticks, is a significant pathogen affecting equine health. This study aimed to investigate the potential impact of *T. equi* infection on Arabian stallion fertility by assessing serological and hormonal changes. A total of 100 Arabian stallions in Egypt were screened for *T. equi* infection, and only 30 stallions were found to be positive by microscopic examination (30%) and 39 stallions by cELISA (39%). Microscopic examination detected *T. equi* in lymphocytes and erythrocytes in the infected stallions. Serum analysis revealed significant alterations in hormone levels associated with fertility. Both total and free testosterone levels showed a significant decrease in the serum of infected stallions compared to non-infected individuals. This decrease in testosterone levels could potentially impact various aspects of reproductive function in stallions. Thyroid hormone levels in *T. equi* infected stallions showed a decrease in triiodothyronine (T3) and an increase in thyroxine (T4), crucial for metabolism and reproductive function. Infected stallions show increased serum cortisol levels, indicating stress response activation, which can negatively impact reproductive function and fertility in stallions. Therefore, these findings suggest a potential link between *T. equi* infection and impaired fertility in Arabian stallions. The observed decrease in testosterone (total and free) and tri-iodothyronine (T3) hormone levels, along with the increased thyroxine (T4) and cortisol hormone levels in infected animals, may contribute to compromised reproductive performance. These findings highlight the importance of considering *T. equi* infection as a potential factor in assessing and managing fertility issues in Arabian stallions.

Key words: *T. equi,* Arabian Stallion, Seroprevalence, Hormonal Perspectives, Fertility

INTRODUCTION

Theileria equi, a protozoal parasite transmitted by ticks, has emerged as a significant pathogen affecting equine health worldwide (Maurer 1962; De-Waal 1992). In Egypt, the prevalence of *T. equi* varies depending on the age, sex, months, seasons, and geographic areas of the horses (Salib et al. 2013). The best option for diagnosing a chronic *T. equi* infection is the cELISA method, which is more sensitive than any other technique (Cunha et al. 2006; Hall et al. 2013). A *T. equi* infection can cause a variety of clinical symptoms, such as organ damage, fever, and anemia (De-Waal 1992). While the impact of this parasite on the overall health of horses has been extensively studied, its effects on reproductive function, particularly in stallions, have received limited attention. Fertility is a critical aspect of the equine breeding program, and any disruption in reproductive function can have substantial economic and practical implications for the equine industry (Wise et al. 2014). Therefore, understanding the potential influence of the *T. equi* infection on stallion fertility is of vital importance.

Parasites like *Toxoplasma gondii* lower testosterone levels in chronic toxoplasmosis patients, which may impact men's reproductive characteristics (Eslamirad et al. 2013). *Theileria orientalis* infection in bulls showed a significant reduction in libido; these effects could reduce conception rates in an extensive herd situation (Gibson et al., 2020). Male dog infertility is caused by elevated testosterone levels; affection is attributed to external tick infection and blood parasite infection (Babesia spp.) (Abd EL Maged et al. 2021).

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Thyroid and cortisol hormones serve as important indicators for theileriosis, providing insights into the physiological and immunological responses of the host during infection (Vidhyalakshmi et al. 2019). Tri-iodothyronine (T3) and thyroxine (T4), in particular, are two thyroid hormones that are essential for controlling metabolism, growth, and development. During *T. equi* infection, the host's immune system mounts a response to combat the parasite, leading to alterations in thyroid hormone levels (Breuhaus et al. 2006). Theileriosis-induced changes in thyroid hormones can impact various physiological processes, including energy metabolism, thermoregulation, and reproductive function. Monitoring thyroid hormone levels can provide insights into the extent of the infection's impact on the host's metabolic and hormonal balance. Cortisol is a hormone released by the adrenal glands in response to stress. During the *T. equi* infection, the host's immune system is activated, triggering an inflammatory response and immune cell activation. This immune response can lead to increased stress levels in infected animals, resulting in elevated cortisol levels (Cordero et al. 2012; Suarez-Esquivel and Castro-Ramirez 2016).

Therefore, this study aimed to investigate the potential impact of *T. equi* infection on Arabian stallion fertility by assessing serological and hormonal changes.

MATERIALS AND METHODS

Ethical approval

This investigation was conducted according to standard protocols, with no pain or injury to the horse. Additionally, the experiments' procedures were approved by the Ethics Committee of the Veterinary Faculty, Benha University, Egypt (BUFVTM 08-10-23).

Animals and location

This study was conducted from September 2022 to August 2023 with 100 Arabian stallions whose ages ranged from 3 to 15 years old in Egypt (Cairo). Stallions were kept in individual stables with rice straw beds, water, and mineralized salt. Feeding with forages three times daily and grains twice daily. The stallions were exposed to natural light, and they were set free in the yard daily. All stallions were used in breeding and had deworming and vaccination programs. Clinically, almost all horses were emaciated and exhausted while others were healthy. In addition, horses had a history of tick attacks.

Blood collection and microscopic examination

One hundred blood samples were taken from the jugular vein of each stallion, and each sample was put into two sterile tubes. One tube containing EDTA was immediately used for a blood smear, creating a thin smear that was stained with Giemsa, fixed with methanol, and then inspected for *Theileria equi* infection under a light microscope.

In the second tube without anticoagulant, the blood was left at room temperature to coagulate, and after centrifuging serum samples for ten minutes at 3000rpm, they were kept at -20°C until serological analysis (cELISA). After the detection of *Theileria. equi* from each horse, we divided stallions into two groups: the first group was the infected group (n=30) (positive *T. equi* by microscopic and cELISA analysis), the second group was the healthy group (n=10) (negative *T. equi* by microscopic and cELISA analysis), and then we evaluated reproductive hormones (total testosterone and free testosterone), thyroid hormones (T3 and T4) and cortisol for both groups.

Competitive-inhibition ELISA test (cELISA)

Collected serum samples were tested for antibodies against *T. equi* using a competitive enzyme-linked immunosorbent assay (c-ELISA) kit (VMRD, USA).

The test was performed following the instructions of the manufacturer (USDA–APHIS 2005). In this assay, a recombinant *T. equi* equine merozoite antigen 1 (EMA-1)-a coated plate was used, and it used a particular monoclonal antibody (MAb) that recognizes this epitope on the merozoite surface protein. The principle of the assay was to detect the antibodies in serum against the antigen-coated plate by inhibiting the primary monoclonal antibody. The absence of *T. equi* antibodies in the sample serum was detected by little or no inhibition of primary monoclonal antibody binding and indicated by strong color developments. The presence of *T. equi* antibodies in the sample serum is indicated by the weak color developments brought on by the suppression of primary monoclonal antibodies binding to an antigen on the soled face. Using a microplate reader (Expert plus UV-G020 151- Austria), the mean optical density (OD) at 620 nm was calculated for each well. Results were expressed as a percentage of inhibition (%I) applying the formula:

$$\% I = 100 [1- (\text{Sample O.D.} \div \text{NC O.D.})].$$

The interpretation of results: if %I >40, the samples were considered positive, and if %I <40, the samples were considered negative.

Total testosterone and free testosterone determination

The DRG Testosterone ELISA kit (EIA-2924) and the DRG Free Testosterone ELISA kit (EIA-1559) were used to perform the serum Total Testosterone and Free Testosterone assays in accordance with the manufacturer's instructions. Every standard, sample, and control was carried out twice. The concentrations of testosterone and free testosterone in the samples were then determined, and a standard curve was created.

Triiodothyronine (T3) and Thyroxine (T4) measurements

Serum T3 and T4 concentrations were determined using the Electrochemiluminescence Immunoassay (ECLIA) method, which was carried out on a fully automated analyzer (Cobas e411 immunoassay analyzer) and was designed for use with the Elecsys T3 reagent kit and T4 reagent kit supplied by Roche Diagnostics GmbH (Mannheim, Germany). An electrochemiluminescence signal is observed when a voltage is applied. The testing was carried out in compliance with the manufacturer's guidelines.

Cortisol determination

The DRG Cortisol ELISA (EIA-1887) was used to perform the serum cortisol assay in accordance with the manufacturer's instructions. Every control, sample, and

standard was run in duplicate. Ultimately, a standard curve was created and the samples' cortisol concentrations were determined.

Statistical analysis

The student's "t" test was used to statistically assess data on reproductive hormones (total testosterone and free testosterone) determination, thyroid hormones (T3 and T4), and cortisol (Snedecor and Cochran 1994). The acquired outcomes were presented as Mean±SE. P<0.05 was recognized as a statistically significant value.

RESULTS

Microscopic and serological (cELISA)

Of the 100 blood samples from Arabian stallions that tested for *T. equi* infection, 30 (30/100) were positive by microscopic examination, and 39 (39/100) were positive by cELISA. The prevalence of *T. equi* by the cELISA assay was significantly higher (P<0.001) than the microscopic examination (ME) (Table 1).

Table 1: The prevalence of *T. Theileria equi* infection in Arabian stallions by ME & cELISA

Detection methods	No. of examined stallions	No. of Positive samples	No. of negative samples	Prevalence %
Microscopic examination	100	30	70	30
cELISA	100	39	61	39***

***Subscript significance difference (P<0.000) between the same columns

Intra-erythrocytic, relatively small, less than 2µm long, pear- and ring-shaped *T. equi* merozoites were observed by microscopic examination of blood smears from infected stallions and stained with Giemsa. The "maltese cross" tetrad is formed by these four pear-shaped *T. equi* merozoites in the erythrocytes. Lymphocytes revealed theilerial schizogony forms, which are irregularly shaped structures (microschizonts or macroschizonts) that contain small chromatin granules (Fig. 1A & B and Fig. 2C & D).

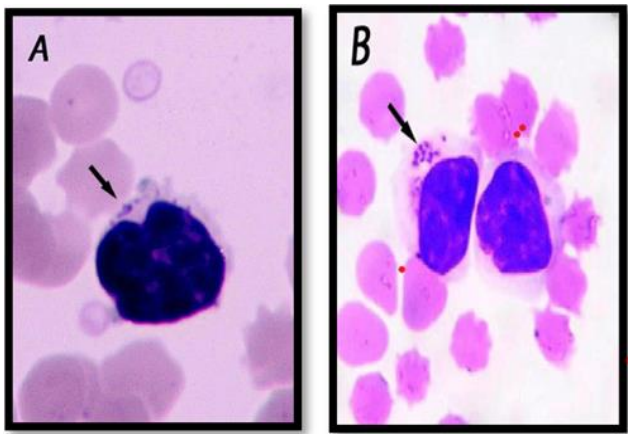

Fig. 1: A and B) Theilerial schizogony forms in lymphocytes in a blood smear of an equine infected with Theileria equi. Giemsa is Stained.

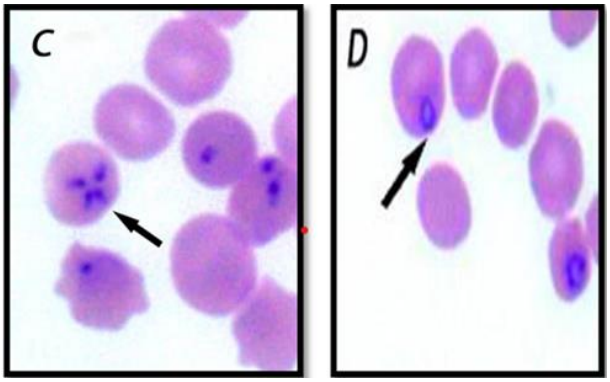

Fig. 2: C) Maltese cross shape, and D) ring shape merozoites blood smear of an equine infected with *Theileria equi*. Giemsa Stain.

Total testosterone and free testosterone

The levels of total testosterone and free testosterone showed a significant (very strong) decrease (p<0.001) in Arabian stallions infected with *T. equi* in comparison with healthy Arabian stallions. As total testosterone and free testosterone levels are related to fertility, a significantly decreased level reflects a decrease in fertility performance (Fig. 3 and 4; Table 2).

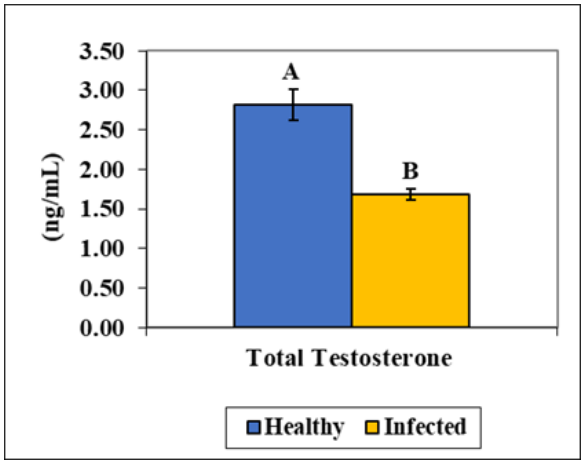

Fig. 3: Total testosterone levels in healthy and *Theileria equi* infected Arabian stallions.

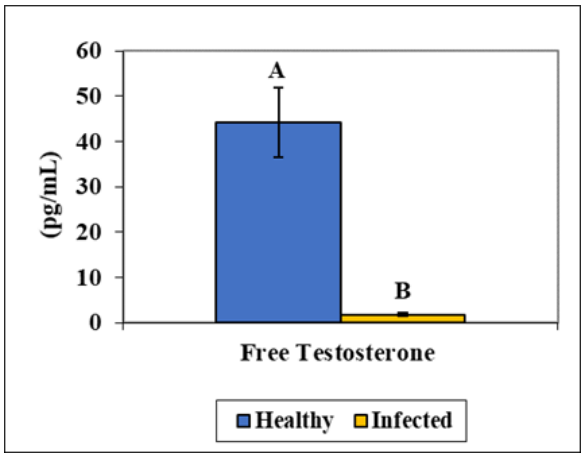

Fig. 4: Free testosterone levels in healthy and *Theileria equi* infected Arabian stallions.

Table 2: Total Testosterone, Free Testosterone, T3, T4, and Cortisol in Arabian stallions infected with *Theileria equi* and healthy Arabian stallions

Parameters	Groups		P Value
	Healthy (A) (n=10)	Infected (B) (n=30)	
Total Testosterone (ng/mL)	2.82±0.20 a	1.68±0.07 b	0.000
Free Testosterone (pg/mL)	44.17±7.79 a	1.80±0.26 b	0.000
T3 (ng/dL)	161.15±5.00 a	121.68±2.59b	0.000
T4 (µg/dL)	3.71±0.23 b	4.47±0.13a	0.007
Cortisol (ng/mL)	43.10±1.80 b	49.30±1.00a	0.003

Values (Mean±SE) bearing different letters in a row differ significantly (P<0.01).

Triiodothyronine (T3), Thyroxine (T4) and Cortisol

When compared to healthy Arabian stallions, the T3 level was found to be significantly (very strongly) (P<0.001) decreased in Arabian stallions infected with *T. equi.* Therefore, compared to healthy Arabian stallions, Arabian stallions infected with *T. equi* had a significantly (strongly) increased T4 level (P<0.01). When compared to healthy Arabian stallions, the cortisol level in Arabian stallions infected with *T. equi* was shown to be significantly (strongly) increased (P<0.01) (Fig. 5-7; Table 2).

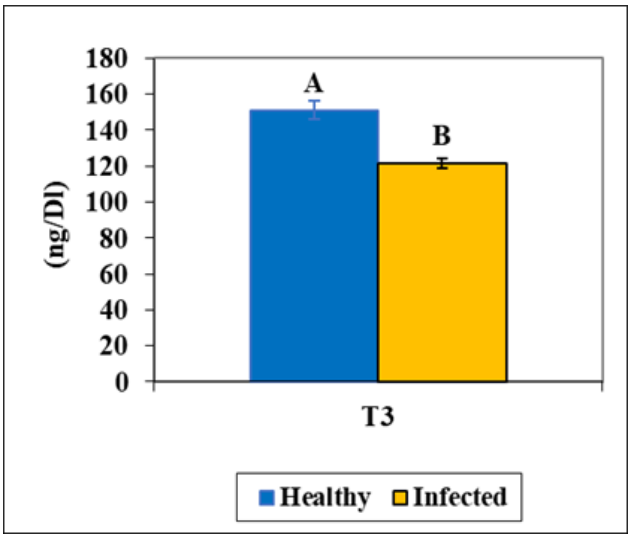

Fig. 5: Triiodothyronine (T3) levels in healthy and *Theileria equi*-infected Arabian stallions.

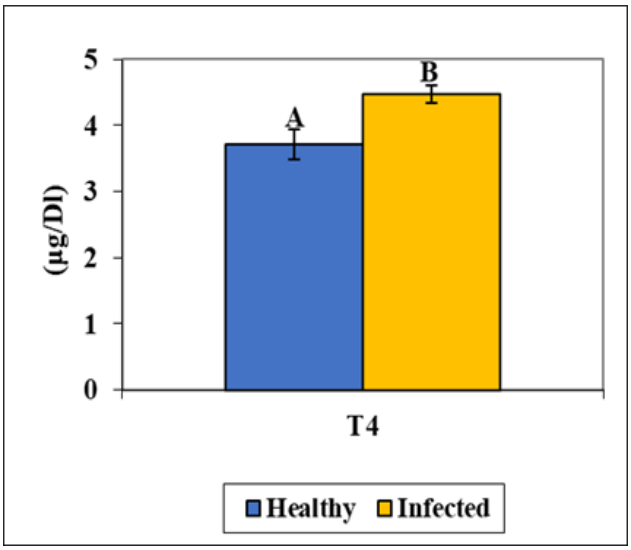

Fig. 6: Thyroxine (T4) levels in healthy and *Theileria equi*-infected Arabian stallions.

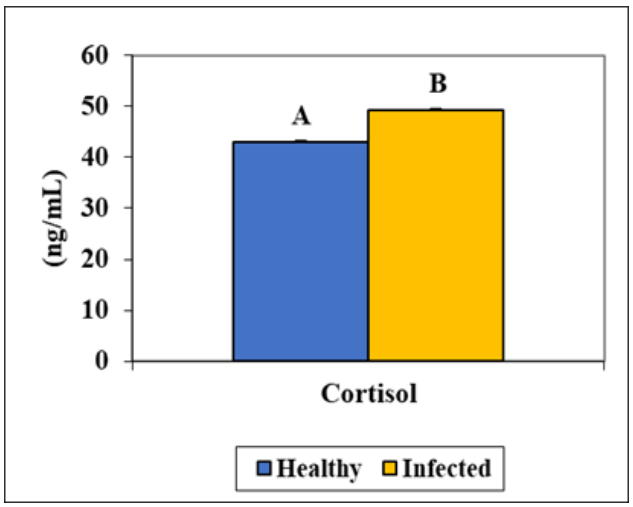

Fig. 7: Cortisol levels in healthy and *Theileria equi* infected Arabian stallions.

DISCUSSION

The Arabian horse is among the oldest and most popular equine breeds worldwide. The Egyptian Arabian stallion breed is also an important source of breeding the best, purest, most beautiful, and most expensive horse breed in the whole world; otherwise, it is used to improve other breeds. *Theileria equi* is a tick-borne blood parasite disease distributed worldwide and endemic in Egypt, according to OIE (2021), as it is responsible for piroplasmosis (theileriosis) in Arabian stallions in Egypt, which has an effect on stallion performance and prevents export to some countries' conditions, a serological analysis for *T. equi*.

This investigation aims to find the prevalence of T. equi in Arabian stallions, a high-value breed in Egypt, using microscopical examination and cELISA, respectively, and compare the prevalence between them. It also aims to evaluate the effect of *T. equi* on the fertility of the Arabian stallion through testosterone hormone determination and the performance affected by thyroid hormones and cortisol determination.

The prevalence of *T. equi* infection in this study 30% and 39% in Arabian stallions in Egypt by ME and cELISA was higher than those recorded in other studies in equine in Egypt and some other countries, as it was in previous studies in general without specific for age, sex, or species. By ME, the prevalence of *T. equi* in this study (30%) was higher than that recorded in Egypt by El-Kelesh et al. (2012) (11.2% for horses), Mahdy et al. (2016) recorded 27.4% for horses, and Kuraa and Nageib (2017) recorded 14% for donkeys. In comparison with some other countries, the prevalence in this study by ME was also higher than

that recorded in Ethiopia, which was 12.2% by Gizachew et al. (2013); in Netherlands, Rampersad et al. (2003) recorded 9.5% prevalence; and in Costa Rica 24.6% prevalence was recorded by Posada-Guzmán et al. (2015). However, the percentage of infection with *T. equi* by using cELISA in the present study (39%) was higher than that recorded in Egypt by El-Kelesh et al. (2012) (13.75% for horses), Mahdy et al. (2016) recorded 15.5% for horses, and Mahmoud et al. (2016) detected 14.8%. In comparison with some other countries, the prevalence in this study by cELISA is also higher than in some other countries studies, as in Jordan by Abutarbush et al. (2012) (14.6%), in Dubai by Jaffer et al. (2010) **(**32.4%), and in Greece by Kouam et al. (2010) (11%).

The prevalence of *T. equi* has been found in our study to be higher than in other studies, which may be because it was examined in adult-age horses only (starting from 3 years old to 15 years old), and that was reported in studies that found *T. equi* persists with increasing age (Moretti et al. 2010; Kouam et al. 2010); otherwise, age of the host and *T. equi* infection were found to positively correlate (Rüegg et al. 2007; Moretti et al. 2010). According to Roberts et al. (2001), sex hormone levels have been linked to susceptibilities to *T. equi* infection, mice used in experiments showed that higher testosterone levels made them more susceptible to piroplasmosis infection and tick infestations (Hughes et al. 2001a, b), the incidence of *T. equi* in stallions in the current investigation may be correlated with sex hormone levels. Breeds have been shown to affect the rate of piroplasmosis seropositivity in some studies (Moretti et al. 2010), so there may be a relationship between the Arabian horse breed and the rate of seropositivity.

In general, the prevalence in our study was higher than other studies recorded in Egypt, possibly as a result of some factors including age which was in adult horses (from 3 to 15 years old), sex which was with stallions, and breed which was with Arabian horses in Egypt. In our study, the prevalence with the cELISA test was higher than the ME test, and that was recorded in almost all studies, as sensitivity was higher with the cELISA test than with the ME test in equine piroplasmosis detection (OIE 2021; Onyiche et al. 2019).

Because testosterone can enhance sexual activity (raising libido and desire), it has a significant impact on fertility (Eisenegger et al. 2017). Total testosterone and free testosterone levels in our study were declining in the Arabian stallions infected with *T. equi* in comparison with healthy Arabian stallions, and this recording is the first in Arabian stallions infected with *T. equi*. In male humans, there was a study in chronic Toxoplasmosis about testosterone levels that recorded a decrease in testosterone levels with *Toxoplasma gondii* (Eslamirad et al. 2013). However, this study is consistent with our study to decreased testosterone levels and blood parasite infection. In male dogs, Abd EL Maged et al. (2021) recorded a highly significant decrease in testosterone levels with babesiosis, and this result is consistent with our study result in testosterone levels affected by decline with blood parasite infection. In bulls, a study on Theileria showed a decrease in bull libido infected with *Theileria orientalis* (Gibson et al. 2020); otherwise, in our study, a decrease in testosterone may affect libido, and in both studies, fertility decline was the final result. Reduced red cell deformability due to *T. equi* infection conformational shift could result in decreased blood flow in the microvasculature (Wise et al. 2014), and that explains our study result about decreased testosterone levels, which may be caused by decreased blood flow to horse testes and so decreased testosterone hormone secretion. According to Donnellan and Marais (2009), severe equine piroplasmosis can cause hypercoagulability, systemic inflammatory response syndrome, and eventually malfunction of many organ systems. According to Ubah et al. (2019), canine babesiosis is the most prevalent cause of infertility in male dogs. In chronic cases, it reduces blood supply, which causes testicular necrosis and hinders the process of spermatogenesis, which explains the decrease in testosterone levels with *T. equi* infection in stallions.

Triiodothyronine (T3) hormone was decreased, and Thyroxine (T4) hormone was increased in stallions infected with *T. equi* (in this study); these recorded thyroid hormone levels agreed with Vidhyalakshmi et al. (2019) (equine study); otherwise, in bovine theileriosis, thyroid hormones were affected (Khalil et al. 2011; Sangwan et al. 2002). *Babesia Canis* infected dogs showed lower thyroid hormone concentrations, according to Kučer et al. (2019), and explained this reduction by decreased albumin and transthyretin. There are several causes of the hypothalamic TRH shortage that lead to hypothyroidism, including decreased caloric intake, low leptin levels during starvation, and activation of inflammatory cytokines (Feldman and Nelson 2003). According to Adler et al. (2007), the organism tries to conserve energy by minimizing catabolism during periods of extreme stress, starvation, or serious illness, which is why basal T3 levels are reduced. Hyperthyroidism can be present normally; however, total T4 concentrations are normal to increased, but concentrations of free T4 are markedly increased in cases of tumours, which are almost always non-functional adenomas that do not progress into adenocarcinomas or impair thyroid gland function**;** otherwise, it is common for older horses to develop thyroid tumours or in chronic disease due to inflammation of the thyroid gland (Dalefield and Palmer 1994; Alberts et al. 2000; Tan et al. 2008).

The cortisol level was increased in the present study, and that recording agrees with Vidhyalakshmi et al. (2019). In comparison with other animal studies, such as in dogs, it corresponds to our study (van Zyl et al. 2023). Cortisol has an important role in equine endocrinology through the integral endocrine response to stress. Increased glucocorticoid production (Bethin et al. 2000; Franchimont et al. 2002) combined with impaired metabolism, which results in a prolonged cortisol half-life (Boag et al. 2020; Boonen et al. 2013), is thought to be the multifactorial mechanism underlying increased cortisol concentration during critical illness, so in the present study, increased cortisol levels in stallions infected with *T. equi* may be caused by stress responses.

Conclusion

The prevalence of *T. equi* in Arabian stallions in Egypt is higher in this study than in previous studies, which may be due to age, sex, or breed factors. *T. equi* infection decreases testosterone (total and free) and triiodothyronine (T3) hormone levels, increases thyroxine

(T4) and cortisol hormone levels, and leads to decreased fertility in Arabian stallions.

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Conflict of interest

The authors affirm that they do not have any conflicts of interest.

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Authors' contribution

Omaima Kandil designed the experiment and supported the chemicals and facilities, Noora Thabet brought the samples and did lab work, Omaima Kandil, Noora Thabet, and Khalid Fararh wrote the main text manuscript and analysis of data.

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