Morphological and molecular studies on cerebral and non-cerebral coenurosis in sheep and goats

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Contents

1 Introduction

The term “non-cerebral coenurosis”, also known as “muscular coenurosis”, refers to the occurrence of coenurus cysts in locations of the host other than the brain and spinal cord (Schuster et al. 2010; Christodoulopoulos et al. 2013; Oryan et al. 2014; Christodoulopoulos et al. 2015; Christodoulopoulos et al. 2016). The term “non-cerebral coenurosis” has been created in contrast to the term “cerebral coenurosis” or “coenurosis” that refers to the occurrence of coenurus cysts, usually in the brain and rarely in the spinal cord, of many mammalian species, including humans, but especially sheep (Hall 1910; Soulsby 1982; Ing et al. 1998; Sharma and Chauhan 2006; Christodoulopoulos 2007; Christodoulopoulos et al. 2008).

Cerebral coenurosis (gid or sturdy) is caused by *Coenurus cerebralis*, the larval or so-called bladderworm stage of *Taenia multiceps* (Leske 1780; Syn. *Multiceps multiceps*) (Soulsby 1982). The current literature, however, is not clear about the species of parasite responsible for “non-cerebral coenurosis” in sheep and goats. Hall (1920) reported that the early stages of *Coenurus cerebralis*, which do not reach the central nervous system, begin development but very soon die and degenerate.

Non-cerebral coenurosis was first described in sheep by Benkovskij (1899) and later in goats by Gaiger (1907). The parasite responsible for non-cerebral coenurosis was initially named *Multiceps gaigeri* by Hall (1916) for goats and *M. skrjabini* by Popov (1937) for sheep.

When first described, both *M. gaigeri* and *M. skrjabini* were considered distinct from one another and from *M. multiceps*. The genus *Multiceps* was later considered a synonym of the genus *Taenia* (Baer 1926; Ortlepp 1938; Abuladse 1964; Verster 1969; Loos-Frank 2000). Verster (1969), in her important revision of the genus *Taenia*, considered *T. gaigeri* and *T. skrjabini* as synonyms of *T. multiceps*. According to Verster (1969), differences in the niche inside the host for cases of *T. multiceps and T. gaigeri* were associated with the species of the intermediate host: coenuri mature only in nervous tissue in sheep but may reach maturity in other organs

in goats. Verster (1969) stated that *T. skrjabini* was probably a subspecies of *T. multiceps*, and the differences in niche were due to isolation and selection in a restricted geographic locality (Kazakhstan). Modern studies adopted Verster's opinion, and *T. gaigeri* was considered a synonym of *T. multiceps*, and *T. skrjabini* was abandoned (Soulsby 1982; Loos-Frank 2000; Smith and Sherman 2009).

Interest in non-cerebral coenurosis has increased since 2010 (Oryan et al. 2010; Schuster et al. 2010; Afonso et al. 2011; Varcasia et al. 2012; Christodoulopoulos et al. 2013; Akbari et al. 2015; Amrabadi et al. 2015; Christodoulopoulos et al. 2015; Schuster et al. 2015; Christodoulopoulos et al. 2016). Cases of non-cerebral coenurosis constitute a problem of food hygiene due to their intramuscular location; carcasses infected with such parasites are rejected during meat inspection. The disorder is important for the sheep and goat industry in Africa and southern Asia, where cases are quite frequent. Non-cerebral coenurosis, however, has never been described from sheep or goats in Europe or the Americas (Schuster et al. 2010; Afonso et al. 2011; Christodoulopoulos et al. 2013; Christodoulopoulos et al. 2015; Schuster et al. 2015).

Schuster et al. (2010, 2015) described non-cerebral coenurus cysts in various muscles and attached to the kidneys and omentum in goats slaughtered in the United Arab Emirates. Afonso et al. (2011) described cerebral and non-cerebral coenurus cysts in goats in Mozambique. Molecular studies of two mitochondrial genes [NADH dehydrogenase subunit 1 (*nad*1), cytochrome c oxidase subunit 1 (*cox*1)] from non-cerebral coenuri in goats supported the opinion that *T. gaigeri* was a synonym of *T. multiceps*, and only intraspecific variation was noted between non-cerebral coenuri and *T. multiceps* (Oryan et al. 2010; Varcasia et al. 2012).

Akbari et al. (2015) more recently described the experimental reproduction of adult taenias from protoscolices of coenurus cysts collected from the brains of sheep and the muscles of goats from the same geographical area of southern Iran. The authors concluded that the same taenias produced both cerebral and non-cerebral forms in sheep and goats. This conclusion supported previous data from Iran, where the forms causing non-cerebral coenurosis could also affect the brain to produce cerebral cases (Kheirandish et al. 2012). Furthermore, Amrabadi (2015) demonstrated that non-cerebral cysts from Iranian goats and cerebral cysts from Iranian sheep were 100% genetically identical based on an exonic region of the enolase (ENO) gene and two mitochondrial (*cox*1 and *nad*1) markers.

In contrast to previous studies, our investigation included cases of non-cerebral cysts that originated in a wide range of geographical regions in the tropics (from Bangladesh to Ethiopia) and from different host species (sheep and goats) and were compared with cerebral cysts from the extended area of continental Greece, where cerebral coenurosis is common (Christodoulopoulos 2007; Christodoulopoulos et al. 2008) but where non-cerebral coenurosis has never been described, to the best of our knowledge. Our investigation produced three publications (Christodoulopoulos et al. 2013; Christodoulopoulos et al. 2015; Christodoulopoulos et al. 2016), providing answers to research questions such as:

(i) the presence or absence of non-cerebral coenurosis in sheep (Christodoulopoulos et al. 2013),

(ii) the description of non-cerebral coenurosis in the intermediate host (sheep and goats) (Christodoulopoulos et al. 2013; Christodoulopoulos et al. 2015), and

(iii) the phylogenetic resolution of the *T. multiceps*/*T. gaigeri* cluster and a possible explanation why non-cerebral coenurosis has never been described from some geographical areas (Christodoulopoulos et al. 2016).

2 Publications of cumulative dissertation

2.1. Occurrence of non-cerebral coenurosis in sheep.
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Occurrence of non-cerebral coenurosis in sheep

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Abstract

This study reports seven rare cases of non-cerebral coenurosis in sheep. The sheep were slaughtered in abattoirs of Abu Dhabi (United Arab Emirates) but originated from India, Iran, Oman and Sudan. The prevalence of infection with non-cerebral coenurosis was 0.008%. The locations of the cysts were the triceps brachii muscle, the diaphragm, the infraspinatus muscle of the shoulder, the muscles of the thigh and the abdomen, and the ommentum. The *Coenurus* cysts were surrounded by a fibrous, semi-opaque membrane, cloudy white in colour. Altogether, 12 cysts were recovered and all contained a single bladderworm. Cysts had a volume of $7.3 \pm 1.30\,cm^3$ (ml), with 7.3 ± 4.0 clusters of scolices, and an average number of scolices 75.3 ± 24.4. These features in sheep were similar to those reported for non-cerebral *Coenurus* cysts in goats. No cysts were found in the brain or spinal cord of any of the infected sheep. No clinical evidence of non-cerebral coenurosis had been recorded during the antemortem veterinary inspection of the infected sheep.

Introduction

Coenurosis (gid or sturdy) is caused by *Coenurus cerebralis*, the bladderworm stage of *Taenia multiceps*, which develops predominantly in the brain or spinal cord of many mammalian species, including humans, and especially sheep (Ing *et al.*, 1998; Sharma & Chauhan, 2006; Christodoulopoulos, 2007; Christodoulopoulos *et al.*, 2008). However, there have also been reports of *Coenurus* cysts in subcutaneous or intramuscular tissues and the abdominal cavity in ungulates but especially goats. In goats, these non-cerebral *Coenurus* cysts have been called *Coenurus gaigeri*, and in sheep they have been called *Coenurus skrjabini* (Verster, 1969; Sharma & Chauhan, 2006; Schuster *et al.*, 2010). Meanwhile, the metacestodes of *Taenia serialis* cause cysts in subcutaneous and muscle tissues of hares and rabbits, known as *Coenurus serialis* (Verster, 1969). The objective of the present study was to report the occurrence of non-cerebral coenurosis in sheep and to describe its macroscopic features.

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Materials and methods

Collection and examination of sheep carcasses

An abattoir survey of non-cerebral coenurosis in sheep was performed in three abattoirs in the city of Al Ain of the United Arab Emirates (UAE) between October 2010 and May 2011. During this period, after the customary examination by the meat inspectors, any sheep carcass suspected of displaying non-cerebral

Table 1. Macroscopic characteristics of *Coenurus* cysts found in sheep ($N = 7$).

	Volume of cyst (cm^3)	Number of scolices per cyst	Number of clusters per cyst	Number of scolices per cluster
Mean ± SE	7.33 ± 1.30 ($n = 12$)	75.33 ± 24.43 ($n = 12$)	7.25 ± 4.03 ($n = 12$)	8.83 ± 2.12 ($n = 87$)
Range	1–16	5–357	1–14	1–28

coenurosis was brought to the attention of the authors for further investigation.

Any carcass identified for investigation was inspected by visual examination as well as by palpation. The suspected cyst was revealed by dissecting the surrounding tissue. If a *Coenurus* cyst was suspected, it was removed, placed in a plastic bag that was labelled and transferred in a portable fridge at 4–8°C to the laboratory within 1 h.

The following data were recorded: the age and the country of origin, the position of the *Coenurus* cysts in the body, the number of the cysts, as well as the presence of any other kind of cestode cysts found. The brain and the spinal cord were examined for the presence of any *Coenurus* cysts.

Examination of cysts

A cyst was initially identified as a *Coenurus* cyst based on the inclusion of a bladder that was filled with watery fluid and having a thin and transparent wall with numerous scolices attached to its inner surface. Upon laying the bladderworm on a flat surface, the number of scoleces and their arrangement in clusters were counted.

To confirm the identification, a piece of the larval membrane containing a cluster of scoleces was submerged in warm tap water (37–39°C) for 30–60 min to provoke evagination of the scolices. Subsequently, the cluster was placed on a slide, a cover slip was pressed tightly on it and finally the scoleces of the cluster were examined under a light microscope. The identification of the *Coenurus* larvae was based on the recognition of the rostellar hooks along with the four surrounding suckers in the evaginated scolices.

Results and discussion

Only 7 sheep of 90,415 (0.008%) slaughtered were found to have non-cerebral *Coenurus* cysts. Three sheep originated in India, 2 from Iran, 1 from Oman and 1 from Sudan. All 12 cysts contained a single bladderworm with multiple scolices (table 1). The cysts were located in the triceps brachii muscle (fig. 1), the diaphragm, the infraspinatus muscle, the muscles of thigh and the abdomen, and in one case a *Coenurus* cyst was found attached to the omentum. One cyst of volume 1 cm^3 was found in the subcutaneous tissue of the thigh. In the cases of infestation in the muscles of thigh and the abdomen, the cysts affected more than one muscle (table 2). No clinical evidence for non-cerebral coenurosis

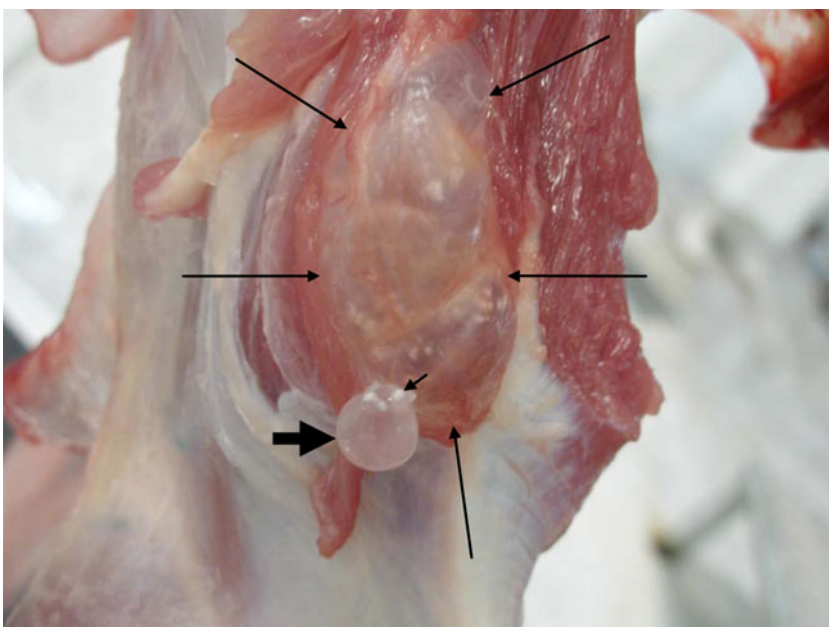

Fig. 1. *Coenurus* cyst attached to the triceps brachii muscle. The outer membrane of the bladderworm was ruptured during skinning so the bladderworm protrudes and a cluster of scoleces is revealed. The margins of the *Coenurus* cyst (size: 7 × 3.86 cm) are indicated by the long, thin arrows, the bladderworm (diameter: 1.38 cm) is indicated by the thick, short arrow, and the cluster of scoleces is indicated by the thin, short arrow (a colour version of this figure can be found online at http://www.journals.cambridge.org/jhl).

Table 2. The occurrence, the age of sheep and the location of *Coenurus* cysts.

Origin of sheep	Age of sheep (months)	Position of *Coenurus* cysts (number of *Coenurus* cysts)
Iran	4	Infraspinatus muscle (1), diaphragm (1)
Iran	>24	Thigh (1)
India	4	Thigh (2)
India	6	Infraspinatus muscle (1)
India	24	Triceps brachii muscle (1), ommentum (1)
Oman	>24	Abdomen (1), ommentum (1)
Sudan	12	Thigh (2)

had been recorded during the antemortem veterinary inspection.

A prevalence of 2.6% has been reported for non-cerebral coenurosis in goats in Iran (Oryan *et al.*, 2010). To the authors' knowledge, no epidemiological data are available for the situation in sheep. The most recent reports of non-cerebral coenurosis in sheep trace back to the 1930s (Schuster *et al.*, 2010). Verster (1969) reported that the disorder occurred only in an isolated area of Kazakhstan; however, the present data suggest that the disorder occurs in Asia, the Middle East and Africa. Interestingly, in the literature there are reports of the occurrence of non-cerebral coenurosis in goats in Asia, the Middle East and Africa (in India, Iran and Oman) (Sharma *et al.*, 1995; Sharma & Chauhan, 2006; Oryan *et al.*, 2010; Schuster *et al.*, 2010).

The *Coenurus* cysts found in the present study were of small size and contained few scoleces in comparison to the cyst size and number of scoleces reported for cerebral coenuri. It is accepted that cerebral coenuri in sheep can reach 95 cm^3 in size and can contain up to 700 scolices (Boev *et al.*, 1964; Schuster *et al.*, 2010). The number of scolices and the size of cysts reported here for sheep non-cerebral coenurosis are similar to the findings for non-cerebral coenurosis of goats reported by Schuster *et al.* (2010), who found cyst sizes to vary between 1 and 40 cm^3 and the number of scolices per cyst to be between 46 and 474.

In conclusion, non-cerebral coenurosis in sheep is a rare disorder. The taxonomic status of the non-cerebral *Coenurus* cysts remains to be determined by molecular techniques.

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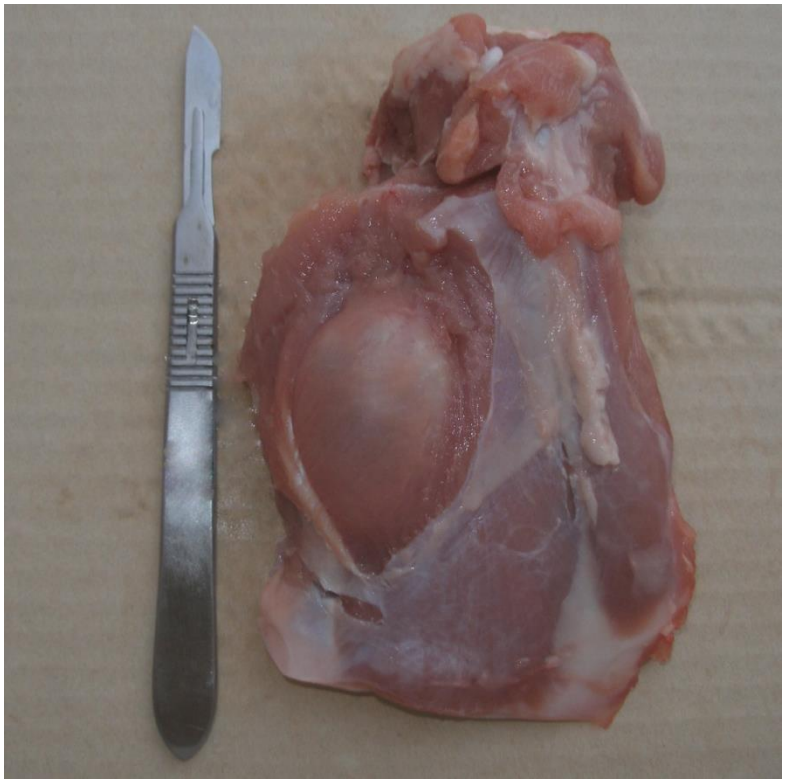
Verster, A. (1969) A taxonomic revision of the genus *Taenia* Linnaeus 1758 s. str. *Onderstepoort Journal of Veterinary Research* **36**, 3–58.

Supplement to paper 2.1.

Fig. S1. Coenurus bladderworm and its surrounding membrane (to the left).

Fig. S2. Coenurus cyst attached to the infraspinatus muscle of the shoulder.



2.2. Characteristics of non-cerebral coenurosis in tropical goats.
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Characteristics of non-cerebral coenurosis in tropical goats

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ABSTRACT

The epidemiological, clinical, and biochemical profile of non-cerebral coenurosis in goats and the morphological characteristics of the responsible metacestodes (cysts) were examined in a cross-sectional survey of slaughtered goats in abattoirs of the United Arab Emirates (U.A.E.) originating from Abu Dhabi and various tropical countries. The age, country of origin, and location of each cyst in the body of goats were recorded. Blood samples collected from infected and matching healthy goats were subjected to biochemical analysis. Data on the morphological characteristics of the cysts as well as the clusters, scoleces, and rostellar hooks in one cyst from each affected carcass were collected. The data collected were subjected to statistical analysis. A total of 2,284 slaughtered goats were examined and 40 goats were diagnosed as infected with non-cerebral coenurus cysts. The prevalence of non-cerebral coenurosis was 1.75% and the degree of parasite aggregation (k) was 0.003, which is indicative of overdispersion ($k < 1$). The only abnormalities observed in the infected goats were palpation of large single cysts in thigh muscles and higher serum aspartate aminotransferase (AST) value. A total of 76 non-cerebral coenurus cysts from 14 different body locations were collected. No cysts were found in the brain or spinal cord. Cysts located in psoas muscles had on average significantly bigger volumes and higher numbers of scoleces and clusters compared to cysts located in other body parts (P-value = 0.000). Significant differences in the morphometric measurements of the rostellar hooks were observed between cysts found in goats from different countries of origin (P-value < 0.05) perhaps due to initial steps of allopatric speciation by geographic isolation. A significant positive correlation was found between number of scoleces and volume of cysts ($b = 6.37 > 5$; R-Sq = 89.4%; P-value = 0.000) and between number of clusters and number of scoleces ($b = 25.13 > 1$; R-Sq = 79.8%; P-value = 0.000) indicative of following a positive allometric growth as well as between number of clusters and volume of cysts ($b = 0.25 < 0.5$; R-Sq = 69.4%; P-value = 0.000) indicative of following a negative allometric growth. The biological significance of the observed allometries is not known, but perhaps for evolutionary reasons the parasite is investing its resources more on the growth of scoleces, less on the growth of cyst volume, and even less on the number of clusters.

1. Introduction

The term "non-cerebral coenurosis" refers to the occurrence of coenurus cysts in body locations of the host other than the brain and the spinal cord. Non-cerebral coenurosis was first described in sheep (Benkovskij, 1899) and then in goats (Gaiger, 1907). The parasite responsible for non-cerebral coenurosis was initially named *Multiceps gaigeri* (Hall, 1916) in goats, and *M. Skrjabini* (Popov, 1937) in sheep (Schuster et al., 2010). However, the later literature considered *M. gaigeri* as the same species with *T. multiceps*, while *M. skrjabini* was rather treated as an unknown entity (Verster, 1969; Soulsby, 1982; Loos-Frank, 2000; Smith and Sherman, 2009).

Recently, the occurrence of non-cerebral coenurosis in sheep has been confirmed (Christodoulopoulos et al., 2013); while the geographical distribution of the disease in both goats and sheep covers a wide range of tropical countries in Asia, Middle East and Africa (Sharma et al., 1995; Sharma and Chauhan, 2006; Oryan et al., 2010; Schuster et al., 2010; Christodoulopoulos et al., 2013). Furthermore, investigation of two mitochondrial genes (CO1 and ND1) supported the opinion that *M. gaigeri* belongs to the same species of

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0304-4017/

T. multiceps and only an intraspecific variation was noted between them (Oryan et al., 2010; Varcasia et al., 2012).

The lack of systematic information on non-cerebral coenurosis in goats was the rationale for undertaking a cross-sectional abattoir survey of non-cerebral coenurus cysts in goats in Abu Dhabi (United Arab Emirates). The objective of the present study was to investigate the epidemiological, clinical, and biochemical profile of non-cerebral coenurosis in goats and the morphological characteristics of the responsible metacestodes (cysts).

2. Material and methods

2.1. Goats

Non-cerebral coenurus cysts were collected from slaughtered goats in three abattoirs namely Bawadi, Falaj-Hazaa, and Yahar in the city of Al Ain of the Abu Dhabi Emirate of the United Arab Emirates (U.A.E.) during January 2011-August 2013. The majority of goats had been imported from various neighbouring countries for slaughtering.

2.2. Ante-mortem clinical examination of goats

All goats were given a full clinical examination before slaughter, including measurements of temperature, heart, and respiratory rate. In addition, the major groups of muscles were palpated in order to detect possible swelling or pain.

2.3. Cyst and data collection

The carcass of slaughtered goats was inspected by visual examination as well as by palpation. The suspected cyst was removed by dissecting the surrounding tissue. If a coenurus cyst was suspected in the carcass, the brain and the spinal cord were also examined visually for the presence of coenurus cysts by dissecting the head and splitting the carcass in half respectively using the saw of the slaughterhouse.

Cysts suspected as coenurus were removed, placed in a labelled plastic bag, and transferred to the laboratory in a portable fridge at 4–8 °C within an hour. In addition, the following data were recorded for goats with suspected coenurus cysts: age, country of origin, and location of each cyst in the body.

2.4. Blood samples

Following the usual ante-mortem clinical inspection of the goats, a 5 ml and a 10 ml blood sample were collected by jugular venipuncture in test tubes with and without heparin respectively. Subsequently, the animals proceeded to slaughtering followed by a detailed post mortem examination by the meat inspector of the slaughterhouse and also by one of the authors (GC or AK). Blood samples from goats found with non-cerebral coenurosis were matched with blood samples from healthy goats of the same batch of animals, same breed, same gender, and approximately same age. The blood samples were subjected to biochemical analysis.

2.5. Biochemical analysis

Blood serum albumin (Doumas et al., 1971) and total proteins (Weichselbaum, 1946) were determined using colorimetric methods. Serum aspartate aminotransferase (AST) (Bergmeyer et al., 1986), gamma-glutamyl transpeptidase (GGT) and creatine kinase (CK) (Szasz et al., 1976) were determined using enzymatic kinetic methods. All measurements were assayed at 37 °C by means of the same spectrophotometer (Shimatzu UV-1601, Tokyo, Japan). In order to exclude the possibility of selenium deficiency which may affect CK, selenium determination was carried out in whole blood by a fluorometric method in a spectrofluorometer (Hitachi Model F-2000) (Christodoulopoulos et al., 2003). The laboratory normal reference values for serum albumin, total proteins, AST, GGT, and CK were 24–44 g/l, 64–78 g/l, 58–350 IU/l, 5–89 IU/l and 20–194 IU/l, respectively. The threshold selenium concentration in whole blood below which goats were considered selenium deficient was 0.07 mg/dl (McComb et al., 2010).

2.6. Examination of cysts

The cysts collected during carcass inspection were examined to confirm their identity and measure their morphological characteristics. A cyst was initially identified as a coenurus cyst when it contained a bladder filled with a watery fluid and having a thin and transparent wall with numerous scoleces attached to its inner surface.

The morphological characteristics of each cyst were measured by establishing its volume by placing it in a measuring cylinder filled with tap water and by laying it on a flat surface and counting the number of scoleces and their arrangement in clusters. As cluster was considered any group of scoleces attached in the cyst membrane in proximity and surrounded by a distinguished area of membrane that was free of scoleces; random, single scoleces surrounded by a distinguished area of membrane free of scoleces were ignored.

For the final confirmation of the identification, a piece of the larval membrane containing a cluster of scoleces was placed on a slide along with some drops of normal saline. A cover slip was pressed tightly on the slide to provoke the evagination of the scoleces and the scoleces of the cluster were examined under a light microscope. The identification of the coenurus larvae was based on the recognition of the rostellar hooks along with the four surrounding suckers in the evaginated scoleces (Soulsby, 1968; Loos-Frank, 2000 Loos-Frank, 2000).

2.7. Examination of rostellar hooks

The rostellar hooks were further examined in one cyst from each affected carcass. For this purpose, five to eight scoleces of each cyst, randomly selected, were cut and placed face down on slides (the top was placed downwards, while the extremity previously attached to the larvae membrane was placed upwards). Some drops of Berlèse solution (cleaning and mounting medium; TCS biosciences, United Kingdom) were added and a cover slip was placed and pressed on each scolex rigorously. The slides were left to dry for 1–2 h and subsequently were observed under a light microscope.

Some of the rostella were posing in the scoleces in such a way that permitted the counting of the total number of small and large hooks (Fig. 1). Only these rostella were used for counting the number of hooks and measuring the dimensions of the rostellar hooks. In case that no rostellum was properly posing, the process was repeated with new scoleces until one rostellum at least from each cyst allowed the proper counting of the hooks.

The measurement of the dimensions of the hooks was accomplished by photographing the rostellum with a digital camera coupled to a light microscope. Measurement of the dimensions was performed using a computerized image analysis system (ImageJ by Softonic®). The accuracy of the measuring method had been previously tested by measuring a scale of known length (10 μm) fixed next to a scolex. Only hooks lying completely 'en face', were measured. Seven morphometric measurements were performed on each scolex: Sum of large and small hooks per scolex, length of large hook, length of handle of large hook, length of blade of large hook,

Table 1
Epidemiological profile and morphological characteristics of non-cerebral coenurus cysts in goats.

Country of origin	Number of goats	Average age of goats in months (range)	Total number of cysts	Mean intensity of cysts/goat (Range)	Body location in goats	Average volume of cysts in ml (range)	Average number of scoleces/cyst (range)	Average number of clusters/cyst (range)
UAE	2	13.0 (2–≥24)	3	1.5 (1–2)	Brachial biceps (1), diaphragm(1), muscles of thigh (1)	6.3 (4–8)	58.7 (23–110)	7 (5–8)
Iran	11	3.8 (2–12)	29	2.6 (1–11)	Abdominal muscles (3), brachial biceps (1), infraspinatus muscle (2), intercostal muscles (1), lumbal muscles (1), muscles of antebrachium (1), muscles of thigh (6), myocardium (1), ommentum (3), pariental pleura (3), perirenal fat (3), psoas muscle (2), triceps brachii muscle (2)	12.4 (1–125)	76.7 (4–782)	7.5 (1–30)
Oman	8	4.5 (2–18)	9	1.1 (1–2)	Abdominal muscles (3), brachial biceps (2), infraspinatus muscle (1), muscles of thigh (1), pariental pleura (1), perirenal fat (1)	5.9 (3–9)	42.1 (16–104)	7 (4–10)
India	8	5.4 (2–≥24)	15	1.9 (1–8)	Abdominal muscles (2), infraspinatus muscle (1), lumbal muscles (2), muscles of thigh (2), myocardium (1), ommentum (2), pariental pleura (1), perirenal fat (1), psoas muscle (1), triceps brachii muscle (2)	10.2 (2–70)	79.3 (5–455)	7.2 (2–24)
Sudan	7	3.0 (2–4)	13	1.9 (1–4)	Abdominal muscles (2), brachial biceps (1), infraspinatus muscle (1), muscles of thigh (3), myocardium (1), ommentum (1), pariental pleura (1), perirenal fat (1), triceps brachii muscle (2)	5.2 (1–13)	32.2 (5–102)	5.7 (1–12)
Yemen	4	11.5 (2–≥24)	7	1.8 (1–4)	Abdominal muscles (1), infraspinatus muscle (1), muscles of antebrachium (1), muscles of thigh (2), ommentum (1), perirenal fat (1)	6.1 (2–15)	45.1 (14–125)	6.3 (1–12)
Total	40	5.4 (2–≥24)	76	1.9 (1–11)		9.2 (1–125)	61.9 (4–782)	6.9 (1–30)

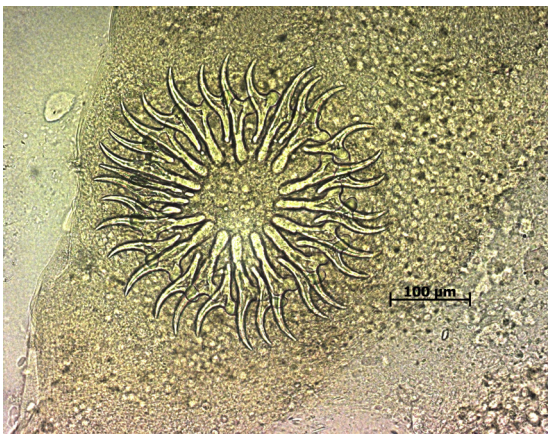

Fig. 1. Large and small rostellar hooks (lateral view).

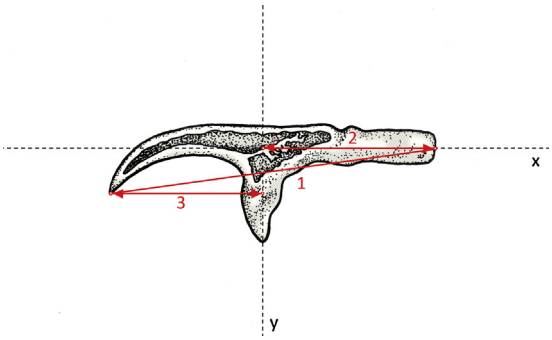

Fig. 2. Parameters used for morphometric measurements of both large and small rostellar hooks: 1-length of hook, 2-length of handle of hook, 3-length of blade of hook. Axis "x" is the midline axis of the hook handle – axis "y" is the vertical axis to the axis "x" passing from the distal point of the hook guard.

length of small hook, length of handle of small hook, and length of blade of small hook (Fig. 2).

2.8. Statistical analysis

The terms used to describe parasite distribution were those of Margolis et al. (1982) where prevalence is the proportion of infected hosts and mean intensity is the mean number of parasites per infected host. The prevalence and the degree of parasite aggregation (k) were estimated as described by Permin et al. (1999).

The data collected were analyzed using descriptive statistics for calculating the averages and standard deviations of continuous variables and the frequencies and percentages of discrete variables. One-way analysis of variance was used to determine the relationship between: (i) cyst characteristics or morphometric measurements of scoleces with origin of animal, (ii) cyst characteristics with cyst position in body, and (iii) infection status of animals with blood biochemical values. Linear regression was used to determine the relationship between cyst characteristics with age of animal and regression analysis was used to determine the relationship between cyst characteristics with morphometric measurements of scoleces.

The growth of the cysts in relation to their scoleces was characterized as isometric, positive, or negative allometric by determining whether the slope (b) of the relative regression line was equal, larger, or smaller than the value of b predicted for isometry. The value of b for isometry was obtained by dividing the rate of change on the y-axis by the rate of change on the x-axis of the regression line ($b = \Delta\tilde{O}/\Delta X$).

The level of significance for the statistical analyses performed was set at 5% and all calculations were conducted using the statistical package Minitab for Windows (Minitab 16, Professional).

3. Results

A total of 2,284 slaughtered goats aged 2 to ≥24 months (average 4.9 months) were examined and 40 goats (Table 1) were conclusively diagnosed as infected with non-cerebral coenurus cysts. The prevalence of non-cerebral coenurosis in the present survey was thus 1.75%. The degree of parasite aggregation (k) was 0.003. Most of the infected goats were 2 month (47.5%) and 4 month (22.5%) old. Only 2 out of the 40 infected goats originated locally from U.A.E., the rest had been imported from Iran (11), Oman (8), India (8), Sudan (7) and Yemen (4).

During ante-mortem clinical examination with palpation a hard mass was detected in the thigh muscles of five young goats. No evidence of pain was noticed during the palpation of the hard mass in these animals. A large coenurus cyst was revealed in each of these five animals during the post-mortem examination with volumes ranging 15–37 cm^3. The coenurus cysts in the remaining animals were not detectable by palpation. No other clinical signs connected to the disease were noticed during clinical examination.

Table 2 shows the results of the blood biochemical analysis of goats infected with non-cerebral coenurosis and uninfected goats. All test values for both infected and uninfected goats were within normal range. No significant differences in test values between infected and uninfected goats were observed except for AST. Infected goats had on average a higher AST value (202.73 IU/l) compared to uninfected goats (141.18 IU/l) (Table 2). Goats with high activity AST had no evidences for any other liver or muscular abnormality. A total of 76 non-cerebral coenurus cysts were collected at the abattoirs from the 40 infected goats. The body location of the cysts were abdominal muscles, brachial biceps, diaphragm, infraspinatus muscle, intercostal muscles, lumbal muscles, muscles of antebrachium, muscles of thigh, myocardium, ommentum, pariental pleura, perirenal fat, psoas muscle, and triceps brachii muscle. No cysts were found in the brain or spinal cord in any of the 40 infected goats (P-value = 0.000). The most frequent body locations of the non-cerebral cysts were muscles of thigh (19.7%) followed by abdominal muscles (14.5%) and omentum (9.2%), while diaphragm (1.3%) and intercostal muscles (1.3%) were the body

Table 2
Blood biochemical profile of goats infected with non-cerebral coenurosis and uninfected goats.

Test	Normal value	Average ± std		P value
		Infected (n = 40)	Uninfected (n = 40)	
Albumin	24–44 g/l	41.35 ± 6.51	42.38 ± 6.33	0.477
Total proteins	64–78 g/l	70.13 ± 5.20	70.43 ± 5.14	0.796
AST	58–350 IU/l	202.73 ± 100.39	141.18 ± 42.52	0.001*
GGT	5–89 IU/l	35.33 ± 7.89	33.78 ± 7.86	0.381
CK	20–194 IU/l	159.75 ± 164.62	108.73 ± 42.54	0.061
Se	>0.07 mg/dl	0.07 ± 0.01	0.07 ± 0.01	0.891

* Significant difference ($p < 0.05$)

Table 3
Frequency of body locations of non-cerebral coenurus cysts.

Body locations of non-cerebral *Coenurus* cysts	Frequency	Percent (%)
Abdominal muscles	11	14.5
Brachial biceps	5	6.6
Diaphragm	1	1.3
Infraspinatus muscle	6	7.9
Intercostal muscles	1	1.3
Lumbal muscles	3	3.9
Muscles of antebrachium	2	2.6
Muscles of thigh	15	19.7
Myocardium	3	3.9
Ommentum	7	9.2
Pariental pleura	6	7.9
Perirenal fat	7	9.2
Psoas muscle	3	3.9
Triceps brachii muscle	6	7.9
Total	76	100.0

locations infected the least (Table 3). One to 11 coenurus cysts per animal were found in the 40 infected goats. The coenurus cysts were surrounded by a strong, semi-opaque membrane, cloudy white in color. All cysts collected contained a single bladder worm with multiple scoleces. Table 1 shows the average and range of their volume, number of scoleces/cyst, and number of clusters/cyst. Table 4 shows the number and length of hooks found in scoleces.

No statistically significant correlation was found between position, volume of cysts, number of scoleces/cyst, or number of clusters/cyst with origin or age of animal. Also, no significant correlation was found between the number of cysts per goat and the seven morphometric measurements performed on each scolex.

On the other hand, a significant positive correlation was found between number of scoleces and volume of cysts ($b = 6.37 > 5$; R-Sq = 89.4%; P-value = 0.000), between number of clusters and volume of cysts ($b = 0.25 < 0.5$; R-Sq = 69.4%; P-value = 0.000), and between number of clusters and number of scoleces ($b = 25.13 > 1$; R-Sq = 79.8%; P-value = 0.000). Furthermore, cysts located in psoas muscles (Fig. 2) had on average significantly bigger volumes and higher numbers of scoleces and clusters compared to cysts located in other body parts (P-value = 0.000).

Significant differences in all seven morphometric measurements of the rostellar hooks were observed between cysts found in goats from different countries of origin. The differences for the total length and the length of handle of large and small hooks had a P-value < 0.001 while these for the length of blade of large and small hooks had a P-value < 0.05.

Finally, significant positive correlations were found between length of large hook (P-value = 0.021; R-Sq = 1.1%), length of handle of large hook (P-value = 0.016; R-Sq = 1.2%) and length of blade of small hook (P-value = 0.036; R-Sq = 1.0%) with age of goats. Some significant positive correlations were also observed among the seven morphometric measurements of the scoleces but which had no meaningful pattern to be listed here.

4. Discussion

Identifying the parasite responsible for non-cerebral coenurosis in the examined goats was outside the scope of the present study. A molecular investigation of the larvae isolated will be published in a following paper.

As mentioned in the introduction, the parasite responsible for non-cerebral coenurosis in goats was initially considered as a separate species named *Multiceps gaigeri* (Hall, 1916). Hall (1910) had already proposed the cestode parasite named *Taenia multiceps* by Leske (1780) to be re-named as *Multiceps multiceps* (Leske, 1780; Hall, 1910 Hall, 1910) in order to restrict the generic name *Taenia* to those forms which have a cysticercus stage in the life history. He found it unnecessary and undesirable to retain in the already large genus of *Taenia* forms having a coenurus or echinococcus larva (Hall, 1910). Finally, Verster (1969) considered *Multiceps multiceps* along with *Multiceps gaigeri* to be synonyms of *T. multiceps. Taenia* species recognised today which have a coenurus stage as larva are *T. multiceps*, *T. serialis* and *T. brauni* (Acha and Szyfres, 2003). Verster (1969) had considered *T. brauni* as a subspecies of *T. serialis.* However, what form constitutes a separate species in the genus *Taenia* is quite complicate task and sometimes even phylogenetic relationships for the recognised species of *Taenia* are estimated differently by morphological and molecular studies (Hoberg et al., 2000; Jia et al., 2010). Meanwhile, current molecular phylogenetic studies propose the division of the genus *Taenia* to three genera: *Taenia*, *Hydatigera* and *Versteria* (Nakao et al., 2013).

The prevalence of non-cerebral coenurosis in the present survey (1.75%) was very different than the prevalence observed in previous surveys of goats in the same area, 0.2% by Schuster et al. (2010) and 16% by Varcasia et al. (2012). The reason for this difference is not known but the prevalence in this kind of surveys is affected by the original composition of each survey's slaughtered animal population. Countries have different levels of livestock hygiene due to the presence of different population sizes of stray dogs, which serve as the main final hosts that spread the infection. In regard to the age, infected goats had a wider range than in the previous studies mentioned above, however the infected goats were younger than 2.5 years old. Schuster et al. (2010) suspected that age is not a limiting factor for infection although he did not report any strong evidence for such hypothesis given that the slaughtered goats are usually young.

An important observation in the present study was that coenurus cysts in the examined goats were aggregated in a few individuals, which is indicative of overdispersion ($k < 1$). Overdispersion is the most common form of frequency distribution of parasitic communities in nature (Bush et al., 2001). Overdispersion is a phenomenon that has been previously described in cestode parasites such as *Hymenolepis citelli* in white-footed deer mouse (Wassom et al., 1986; Munger et al., 1989), *Taenia pisiformis* in dogs (Rashed et al., 1991) and deer mice (Theis and Schwab, 1992), and *Abuladzugnia gutterae, Davainea nana, Hymenolepis cantaniana, Numidella numida, Octopetalum numida, Ortleppolepis multiuncinata, Porogynia paronai, Raillietina angusta, Raillietina pintneri, Raillietina steinhardti, Raillietina* sp. in Helmeted Guineafowls (Junker et al., 2008). Several factors may contribute to overdispersion. Proglottids contain large numbers of infective eggs so their distribution in the environment is aggregated. This aggregation results in numerous eggs being ingested at once when the content of a proglottis is eaten by an intermediate host; obviously the majority of the eggs of each proglottis are released in the environ-

Table 4
Number and length (μm) of hooks found in scoleces ($n = 73$).

	Number of hooks	Measurements of large hooks (μm) ($n = 466$)			Measurements of small hooks (μm) ($n = 432$)		
		Total length of hook	Length of handle	Length of blade	Total length of hook	Length of handle	Length of blade
Average	29.5	155.62	80.08	74.63	113.13	53.95	57.88
Range	24–34	130–177	54–104	59–88	90–140	35–80	47–74

ment in proximity. In addition, climatic variations may result in the environmental aggregation of the eggs because they are not resistant to desiccation so can survive only in soil areas that remain wet (Theis and Schwab, 1992). Finally, the presence of susceptible genotypes in the animal host population (Wassom et al., 1986) and differences in the immune competence and age resistance of individual animal hosts may contribute to overdispersion (Theis and Schwab, 1992).

No differentiating clinical signs of non-cerebral coenurosis were observed during ante-mortem clinical examination. Given the epidemiology of the disease in this area, the detection of a hard mass in thigh muscles of goats, points to a tentative diagnosis of non-cerebral coenurosis.

Infected goats had on average a significantly higher AST, but within normal range, compared to uninfected goats. The average CK was also higher in the infected group, but the difference with the uninfected was not significant. Elevated AST values are indicative of liver or muscle cell damage; while elevated CK values are indicative of muscle cell damage (Smith and Sherman, 2009). Since no liver damage was detected during carcass inspection, the elevated AST and CK values are attributed to muscle damage perhaps due to parasitism. Generally, all blood tests indicate that the animals despite their parasitism were healthy.

Cysts were located in 14 different body locations indicating that the parasite has adapted to living in tissues other than the brain. Cysts located in psoas muscles had on average significantly bigger volumes and higher numbers of scoleces and clusters compared to cysts located in other body parts. The reason for this difference may be related to the less mechanical resistance as the cysts were free to grow into the peritoneal cavity while the same time they were in contact with a specific muscle. Psoas muscle has the longest in sarcomere length, the smallest size of muscle fiber, and the lowest shear force value compared to other muscles (Sitthigripong et al., 2013). It is worth to note that only the small mechanical resistance, which the proximity to the peritoneal cavity offers, is probably not able to increase the size of the cyst. Cysts located in momentum or perirenal fat were found to be of small size (4.786 ± 1.847; range 1–8 ml; $n = 14$).

The number of clusters and scoleces as well as the averages and ranges of the number and the lengths of large and small hooks were similar to those listed in a previous study by Schuster et al. (2010). This similarity indicates that both studies dealt with the same parasite species. The reason why especially the blades of the hooks in cysts from different origin were significantly different in length is not known, but it may be due to initial steps of allopatric speciation by geographic isolation (Huyse et al., 2005).

It is worth mentioning that the number of the rostellar hooks in the different scoleces of the same cyst was in some cases different. In these cases usually the differences in the hook number were one or two couples of hooks. Even though there are no available related data in the literature, it seems that perhaps the exact number of rostellar hooks which each parasite finally develops depends not only on its genetic characteristics but also on other extrinsic factors such as available nutrients (Sopikov, 1931).

Finally, another interesting observation was that the rate of increase of the number of scoleces was proportionally larger compared to the rate of increase in the volume of the cysts ($b = 6.37 > 5$) and compared to the rate of increase in the number of clusters ($b = 25.13 > 1$) indicating in both these cases a positive allometric growth. On the other hand, the rate of increase of the number of clusters was proportionally smaller compared to the rate of increase in the volume of the cysts indicating a negative allometric growth ($b = 0.25 < 0.5$). The biological significance of the observed allometries is not known. However, the propagation of the parasite directly depends on the number of scoleces and therefore, as an evolutionary adaptation, the parasite is investing its resources more on the growth of scoleces than on the growth of cyst volume or on the number of clusters.

In conclusion, non-cerebral coenurus cysts had a low prevalence and an overdispersed distribution in goats following an allometric growth. The most frequent body location of the cysts was the muscles of thigh, but the cysts in the psoas were the most developed. The only clinical and laboratory abnormalities observed in the infected goats were the palpation of large single cysts in thigh muscles and the higher serum aspartate aminotransferase (AST) value.

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Supplement to paper 2.2.

Fig. S1. Large coenurus cyst in thigh, detectable by palpation.

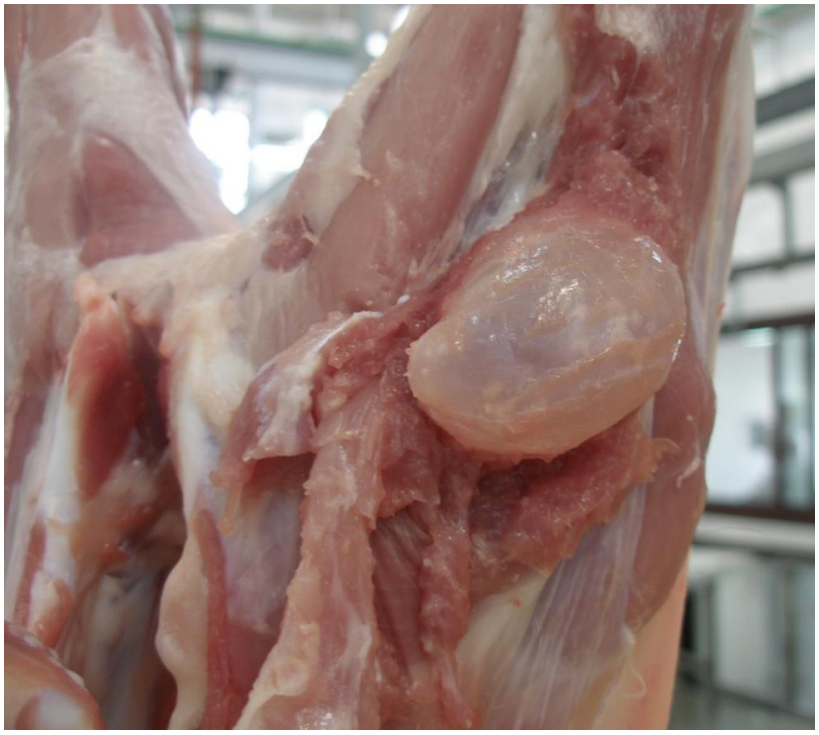
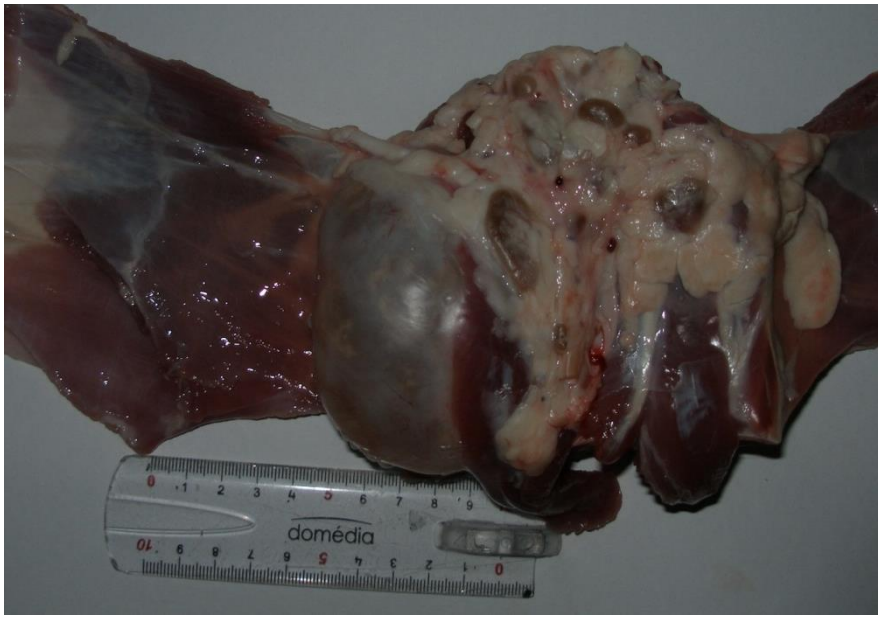
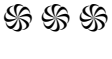


Fig. S2. Coenurus cyst in psoas muscle.





2.3. Cerebral and non-cerebral coenurosis:
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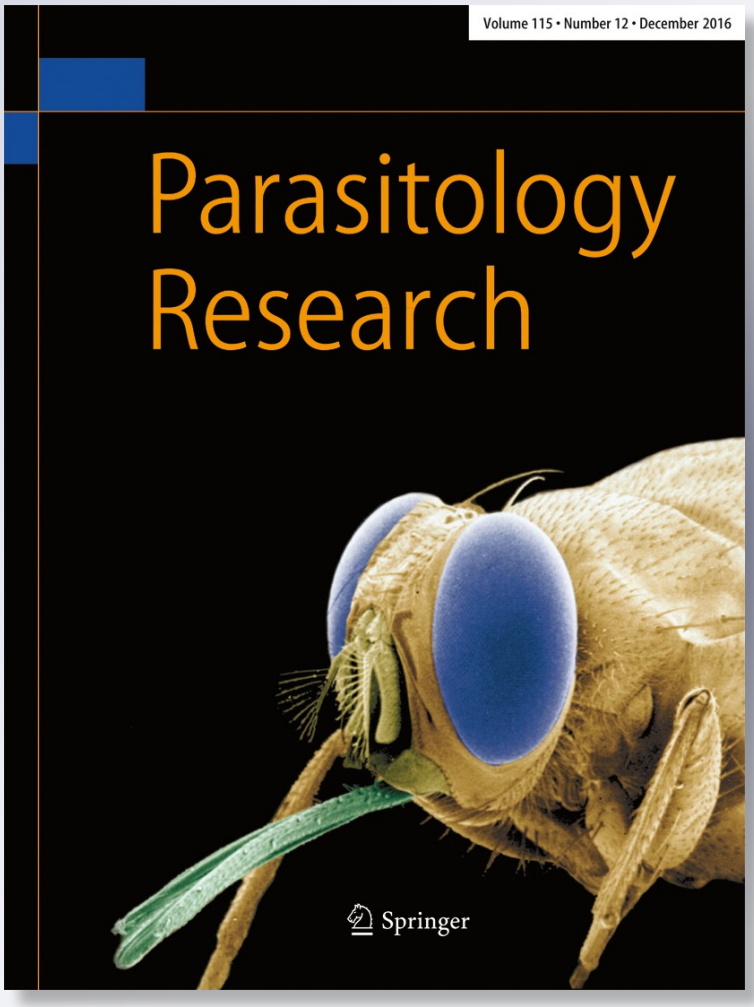

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ORIGINAL PAPER

Cerebral and non-cerebral coenurosis: on the genotypic and phenotypic diversity of *Taenia multiceps*

Georgios Christodoulopoulos[1,2] · **Anke Dinkel**[1] · **Thomas Romig**[1] · **Dennis Ebi**[1] · **Ute Mackenstedt**[1] · **Brigitte Loos-Frank**[1]

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Abstract We characterised the causative agents of cerebral and non-cerebral coenurosis in livestock by determining the mitochondrial genotypes and morphological phenotypes of 52 *Taenia multiceps* isolates from a wide geographical range in Europe, Africa, and western Asia. Three studies were conducted: (1) a morphological comparison of the rostellar hooks of cerebral and non-cerebral cysts of sheep and goats, (2) a morphological comparison of adult worms experimentally produced in dogs, and (3) a molecular analysis of three partial mitochondrial genes (*nad*1, *cox*1, and 12S rRNA) of the same isolates. No significant morphological or genetic differences were associated with the species of the intermediate host. Adult parasites originating from cerebral and non-cerebral cysts differed morphologically, e.g. the shape of the small hooks and the distribution of the testes in the mature proglottids. The phylogenetic analysis of the mitochondrial haplotypes produced three distinct clusters: one cluster including both cerebral isolates from Greece and non-cerebral isolates from tropical and subtropical countries, and two clusters including cerebral isolates from Greece. The majority of the non-cerebral specimens clustered together but did not form a monophyletic group. No monophyletic groups were observed based on geography, although specimens from the same region tended to cluster. The clustering indicates high intraspecific diversity. The phylogenetic analysis suggests that all variants of *T. multiceps* can cause cerebral coenurosis in sheep (which may be the ancestral phenotype), and some variants, predominantly from one genetic cluster, acquired the additional capacity to produce non-cerebral forms in goats and more rarely in sheep.

Keywords *Taenia multiceps* · *Taenia gaigeri* · *Taenia skrjabini* · Cerebral coenurosis · Non-cerebral coenurosis · Muscular coenurosis · Molecular · Mitochondrial genes

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Introduction

The cestode *Taenia multiceps* (Leske 1780; syn. *Multiceps multiceps*) is the causative agent of coenurosis (bladderworm) in sheep and goats. The coenurus cysts typically develop in the brain or—rarely—in the spinal cord of the intermediate hosts, causing cerebral coenurosis ("coenurus cerebralis") (Hall 1910; Soulsby 1982; Ing et al. 1998; Sharma and Chauhan 2006; Christodoulopoulos 2007; Christodoulopoulos et al. 2008). Non-cerebral locations of coenuri (often in the musculature) have also been frequently described (Gaiger 1907; Hall 1916; Abuladze 1964; Oryan et al. 2010, 2014; Schuster et al. 2010, 2015; Afonso et al. 2011; Christodoulopoulos et al. 2013, 2015).

The parasite responsible for non-cerebral coenurosis in goats was initially named *Multiceps gaigeri* (Hall 1916), and corresponding stages in sheep were described as *M. skrjabini* (Popov 1937), with their cysts in the intermediate host called *Coenurus gaigeri* and *Coenurus skrjabini*, respectively (Hall 1916; Schuster et al. 2010). The genus *Multiceps* was later considered a synonym of the genus *Taenia* (Baer 1926; Ortlepp 1938; Verster 1969). Verster (1969) included both *T. gaigeri* and *T. skrjabini* in *T. multiceps*, suggesting that *T. skrjabini* might form a subspecies that had developed in a different habitat in a

restricted geographical area (Kazakhstan). Modern parasitologists have adopted Verster's opinion, with *T. gaigeri* and *T. skrjabini* as synonyms of *T. multiceps* (Soulsby 1982; Loos-Frank 2000; Smith and Sherman 2009). Analyses of two mitochondrial (mt) genes (*cox*1 and *nad*1) from goat non-cerebral coenuri added support to this concept, and variations between non-cerebral coenuri and typical *T. multiceps* were considered to be at an intraspecific level (Oryan et al. 2010; Varcasia et al. 2012; Akbari et al. 2015; Amrabadi et al. 2015).

The present paper comprises three studies: (1) a comparative morphological study of the rostellar hooks of protoscolices of cerebral cysts isolated from naturally infected sheep and goats in Greece and of non-cerebral cysts isolated from sheep and goats slaughtered in the United Arab Emirates (UAE) and Egypt, which mainly originated from various countries of the Middle East, northern Africa, and southern Asia; (2) a comparative morphological study of adult worms produced by the experimental infection of dogs with cerebral and non-cerebral cysts from sheep and goats; and (3) a molecular investigation of the cysts mentioned above; DNA sequence variability was investigated within partial sequences of three mt genes: *nad*1 (NADH dehydrogenase subunit 1), *cox*1 (cytochrome c oxidase subunit 1), and the gene encoding 12S rRNA.

The aim of this study was to contribute to the phylogenetic resolution of the *T. multiceps*/*T. gaigeri* cluster using isolates from different host species from a wide geographical range.

Material and methods

Samples of coenurus cysts

Cerebral coenuri were obtained from 20 sheep and 6 goats diagnosed with cerebral coenurosis between 2011 and 2014 at the Department of Clinical Veterinary Medicine of the School of Veterinary Medicine, University of Thessaly, Greece. The animals originated from 14 counties of continental Greece. In cases of multiple cysts, one coenurus from each affected sheep or goat was examined further.

Non-cerebral coenuri were collected between 2010 and 2014 from 7 sheep and 42 goats in abattoirs in the city of Al Ain of the Abu Dhabi Emirate in UAE and in abattoirs in Toukh, Egypt. Two of the 42 goats originated from the UAE, and the other animals had been imported for slaughter from India (3 sheep and 10 goats), Iran (2 sheep and 9 goats), Sudan (8 goats), Oman (1 sheep and 4 goats), Somalia (1 sheep and 3 goats), Yemen (4 goats), Bangladesh (1 goat), and Ethiopia (1 goat). All these areas are referred to throughout this text as the tropics/subtropics. Again, only one coenurus cyst from each affected sheep or goat was examined further.

In all cases, recovered cysts were placed singly in labelled plastic bags and transferred to the laboratory in a portable refrigerator at 4–8 °C within one hour.

Examination of rostellar hooks

The rostellar hooks of the protoscolices were examined in one cyst from each affected carcass. Five to 8 randomly selected protoscolices from each cyst were excised and placed face down on microscope slides, with the part attached to the cyst facing upwards. A few drops of Berlèse solution (cleaning and mounting medium; TCS Biosciences, Buckingham, UK) were added, and a cover slip was firmly pressed onto each scolex. The protoscolices were allowed to dry for 1–2 h and were subsequently observed under a light microscope. We used only rostella with clearly visible hooks for counting and measuring. If all rostella were improperly positioned, the process was repeated with new protoscolices until at least one rostellum from each cyst had hooks that could be properly counted and measured.

The dimensions of the hooks were measured from digital photomicrographs of the rostellum using a computerised image-analysis system (ImageJ by Softonic, Barcelona, Spain). The accuracy of this method had been previously tested by measuring a scale of known length (10 μm). Only hooks lying on the lateral side (flat) and completely on a horizontal plane were measured. The dimensions of the rostellar hooks can be calculated in various ways (Gubányi 1995). We used seven morphometric measurements for each protoscolex: the numbers of large and small hooks per rostellum, length of the large hook, length of the handle of the large hook, length of the blade of the large hook, length of the small hook, length of the handle of the small hook, and length of the blade of the small hook (Christodoulopoulos et al. 2015).

Experimental production of adult *Taenia* spp.

Six medium-sized mixed-breed puppies were used for the production of adult *Taenia* spp. Each puppy was infected when 5 months old with approximately 30 protoscolices from a single coenurus cyst. Four categories of coenuri were used for infection: (a) cerebral coenuri from sheep (two puppies, each infected with protoscolices from different cysts from different sheep, with both sheep originated in Greece), (b) cerebral coenuri from goats (one puppy; goat originated in Greece), (c) non-cerebral coenuri from sheep (one puppy; sheep originated in Somalia), and (d) non-cerebral coenuri from goats (two puppies, each infected with protoscolices from different cysts from different goats, with one goat originated in Ethiopia and the other one originated in Sudan).

Infections (a) and (b) were done in the Department of Clinical Veterinary Medicine, University of Thessaly (Greece). Infections (c) and (d) were done in the Faculty of Veterinary Medicine, Benha University, Egypt. The experimental puppies were owned by the respective institutions. The experiments were approved by the Animal Ethics Committee of Karditsa County for the experiment in Greece and by the Animal

Ethics Committee of the Faculty of Veterinary Medicine, Benha University, for the experiment in Egypt.

The puppies were maintained in isolated barn cells throughout the experiment and fed on a Purina® Pro Plan® dry diet. Specific care was taken for the disinfection and destruction of their stools. The pre-treatment period began when the puppies were 2 months old. They were then treated with the anthelminthic Drontal® Plus Taste Tabs® (tablet for puppies and small dogs, containing 22.7 mg of praziquantel, 22.7 mg of a pyrantel base as pyrantel pamoate, and 113.4 mg of febantel). The absence of gastrointestinal parasitism was subsequently monitored by weekly faecal examination using Telemann's concentration technique (Linscott and Sharp 2015). Following the experimental infection, the puppies were monitored daily, and their stools were visually inspected for the presence of proglottids and, with Telemann's concentration technique, for the presence of parasitic eggs.

The puppies were humanely euthanised 55 days after the experimental infection using an intravenous injection of 15 ml Dolethal® (VETOQUINOL SA); the puppies weighed 11.2–12.8 kg. The intestine was submerged in a tray with water and opened longitudinally. Worms were removed and counted, the scolices were removed, and the strobilae were washed in water until completely extended. All handling followed special safety precautions because of the possible danger of human infection.

Staining and microscopic examination of *Taenia* spp.

Nine *Taenia* spp. from each puppy were stained and examined further. The scolices were examined as described above ("Examination of rostellar hooks"). The relaxed (extended) strobilae of the *Taenia* spp. were stained in a lactic-acid-carmine solution without prior fixation. After staining, the tapeworms were dehydrated by a graded series of alcohol baths, cleared in wintergreen oil, and mounted in Entellan® new (Merck KGaA, Darmstadt, Germany) (Schmidt 1970).

One specific section of the mounted strobila was measured: the last four consecutive mature proglottids immediately before the beginning of the development of the uterine branches (mature proglottids). A total of 16 morphometric measurements were performed on each examined proglottis (Fig. 1): (1) area of the proglottis, (2) area of the proglottis surrounded by the excretory canals, (3) area of the ovarian lobe on the poral side, (4) area of the ovarian lobe on the non-poral side, (5) area of the vitellarium, (6) length of the cirrus pouch (CP), (7) maximum CP width, (8) length of the vas deferens (VD), (9) maximum VD width, (10) distance between the genital atrium (GA) and the lateral nerve cord, (11) distance between GA and the lateral side of the longitudinal excretory canal (LEC), (12) distance between GA and the medial side of the LEC, (13) distance between GA and the uterus, (14) number of testes on the poral side and posterior to the vagina, (15) number of testes on the poral side and anterior to the VD, and (16) number of testes on the non-poral side. All measurements were performed on digital photomicrographs of the proglottids using a computerised image-analysis system (ImageJ by Softonic, Barcelona, Spain).

Each of the above measurements on the proglottids was the mean of two independent measurements by two researchers. The two researchers were blind to the origin of the *Taenia* spp. For disagreements ≥2 % between the two sets of measurements, the measurements were repeated by the two researchers until the difference was <2 %.

Statistical analysis of morphometric measurements

A one-way ANOVA was used for statistical comparisons of the seven morphometric measurements of the rostellar hooks in the cysts and the 16 morphometric measurements from the mature proglottids between the tissues affected in the intermediate host ("non-cerebral" vs. "cerebral") and their coenurus/host source [parasite source: cerebral coenurus cyst of sheep (*Coenurus cerebralis*/sheep), cerebral coenurus cyst of goat (*Coenurus cerebralis*/goat), non-cerebral coenurus cyst of sheep (*Coenurus skrjabini*/sheep), and non-cerebral coenurus cyst of goat (*Coenurus gaigeri*/goat)]. The level of significance for the statistical analyses was set at 5 %, and all calculations used the statistical package Minitab for Windows, version 17.1 (Minitab Inc., PA, USA).

Molecular analysis

Ethanol-preserved material from all cerebral cysts (20 sheep and 6 goats from Greece) used for the morphometric study was further processed for molecular characterisation; and also a subset of 26 non-cerebral cysts was selected from Iran (2 sheep and 8 goats), India (1 sheep and 5 goats), Oman (1 sheep and 2 goats), Somalia (2 goats), UAE (2 goats), Sudan (1 sheep), Bangladesh (1 goat), and Ethiopia (1 goat).

DNA was extracted by digesting up to 0.5 g of the protoscolices, including the connected cyst wall, with 2 mg/ml proteinase K in 500 μl of 10 mM Tris–HCl (pH 7.5), 10 mM EDTA, 50 mM NaCl, 2 % sodium dodecyl sulphate, and 20 mM dithiothreitol as previously described (Dinkel et al. 1998, 2004). The DNA was purified by phenol–chloroform extraction followed by ethanol precipitation, and the dried DNA was resuspended in 200 μl of nuclease-free water and stored at −20 °C until further use.

Partial DNA sequences of three mitochondrial genes (*nad*1, *cox*1, and 12S rRNA) were amplified using approximately 100 ng of isolated DNA in a total volume of 50 μl. A 471-bp fragment of the *nad*1 gene was amplified by PCR using the primer pair JB11 (5′-AGA TTC GTA AGG GGC CTA ATA-3′) and JB12 (5′-ACC ACT AAC TAA TTC ACT TTC-3′; Bowles and McManus 1993). A 410-bp fragment of

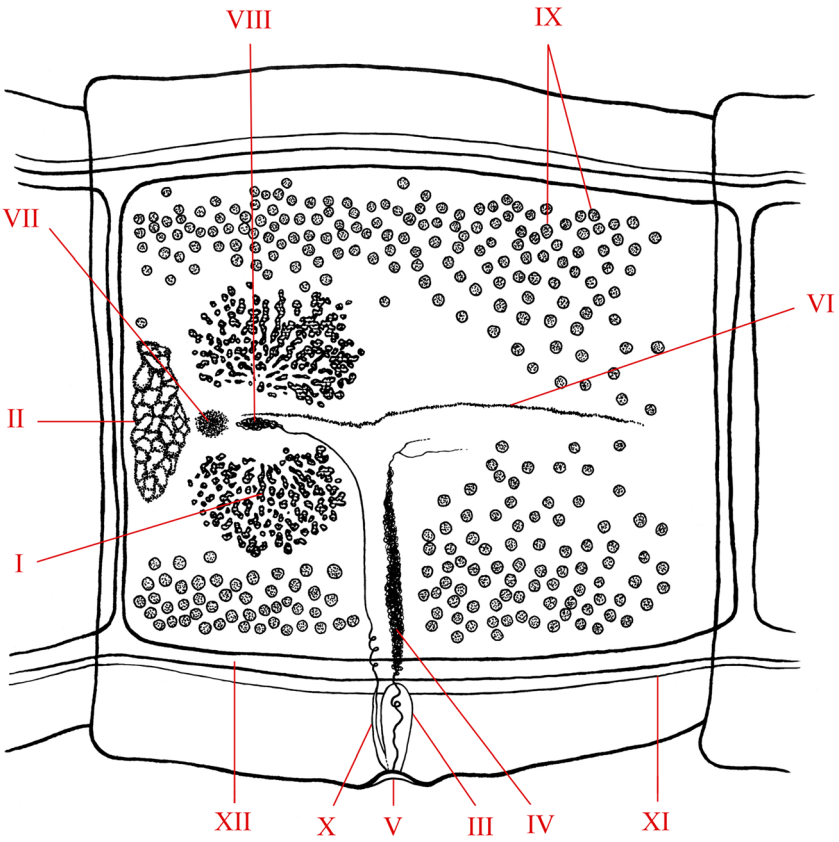

Fig. 1 Mature proglottis of *Taenia multiceps* (*I* ovary, *II* vitellarium, *III* cirrus pouch, *IV* vas deferens, *V* genital atrium, *VI* uterus, *VII* Mehlis' gland, *VIII* receptaculum seminis, *IX* testes, *X* vagina, *XI* lateral nerve cord, *XII* longitudinal excretory canal)

the *cox*1 gene was amplified using the primer pair 2575 (5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′) and 3021 (5′-TAA AGA AAG AAC ATA ATG AAA ATG-3′; Bowles et al. 1992). Both PCRs were performed as described previously (Bowles et al. 1992; Bowles and McManus 1993). A 373-bp fragment of the 12S rRNA gene was amplified using the primer pair P60.for. and P375.rev. as described previously (Dinkel et al. 1998; von Nickisch-Rosenegk et al. 1999) with the following modifications: the 50-μl reaction mixture consisted of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 20 pmol of each primer, and 1.25 units of Ampli-Taq Polymerase (Life Technologies) for 40 cycles (denaturation for 30 s at 94 °C, annealing for 1 min at 55 °C, and elongation for 30 s at 72 °C). After amplification, 10 μl of the amplification products stained with GelRed™ (Biotium, Inc.) was visualised on a 1.5 % agarose gel.

The amplification products were then purified using the QIAquick™ PCR purification kit (QIAGEN, Germany), and the products were sequenced by GATC Biotech AG (Germany) using the corresponding forward and reverse primers. The sequences were analysed using the National Center for Biotechnology Information BLAST programmes and databases. The sequences of the three examined gene parts were concatenated to increase the evolutionary signal, with the order *nad*1-*cox*1-12S rRNA. MultAlin was used to produce multiple sequence alignments (Corret 1988), and the alignments were phylogenetically analysed using MEGA 6 (Tamura et al. 2013) and the nucleotide model HKY+G (jModelTest). Phylogenies were reconstructed using maximum likelihood (ML), and the robustness of the ML trees was evaluated by bootstrapping based on 1000 replicates. The various phylogenetic trees were rooted with *T. serialis*, *T. krabbei*, *T. madoquae*, *T. ovis*, *T. saginata*, *T. asiatica*, *T. solium*, and *T. hydatigena* as outgroups. The network of the concatenated sequences was drawn using TCS 1.2 software (Clement *et al.* 2000) and statistical parsimony (Templeton *et al.* 1992); the network estimation was run at a connection limit of 95 %.

Results

Terms

For brevity, the adult worms that developed from cerebral and non-cerebral coenurus cysts are named "cerebral worms" and "non-cerebral worms", respectively, adding the species of intermediate host if necessary (e.g. "non-cerebral worms/sheep"). The term "parasitic stages" refers to both adult worms and coenurus cysts (e.g. "non-cerebral parasitic stages").

Common features of scolices and hooks

The rostellar hooks have the same arrangement in two circles, typical for the genus *Taenia*, with the tips of the large and small hooks extending to the same circumference and symmetrically surrounded by four suckers. The general morphology of the large and small hooks is the same as described by Hall (1920).

Particularities of the hook shape

In the "cerebral" and the "non-cerebral parasitic stages", the handles of the large hooks commonly end bluntly (Figs. 2 and 3a, b). In the "cerebral parasitic stages", however, the handle sometimes slightly curves dorsally at the distal extremity (Fig. 2). This dorsal curvature is rare and much slighter in the non-cerebral parasitic stages (Fig. 3a). The large notch on the dorsal border of the handle of the large hooks in the non-cerebral parasitic stages is sometimes approximately one third of the handle length and extending to the largest distal part of the handle (Fig. 4).

In the cerebral parasitic stages, the handle of the small hooks is usually curved and only rarely straight; when it is curved, the convexity is on the dorsal side, along most of its length. In addition, the small hooks sometimes have a handle that turns dorsally at its distal extremity. This last feature of dorsal turning on very short handles gives the hooks a specific appearance, where the handle and guard in a lateral view have a sigmoid shape (Fig. 2). In the non-cerebral parasitic stages, the handle of the small hooks is usually straight (Fig. 3a, b) but can show a slight convexity on the dorsal side (Fig. 3a). Cases with a dorsal turn of the distal extremity are rare, and even then the turn is slight (Figs. 3b and 4). We never saw a small hook with a sigmoid-shaped handle and guard in the non-cerebral parasitic stages. The handle of the small hooks is notably significantly shorter in the cerebral stages than in the non-cerebral stages ($p < 0.001$) (Table 1).

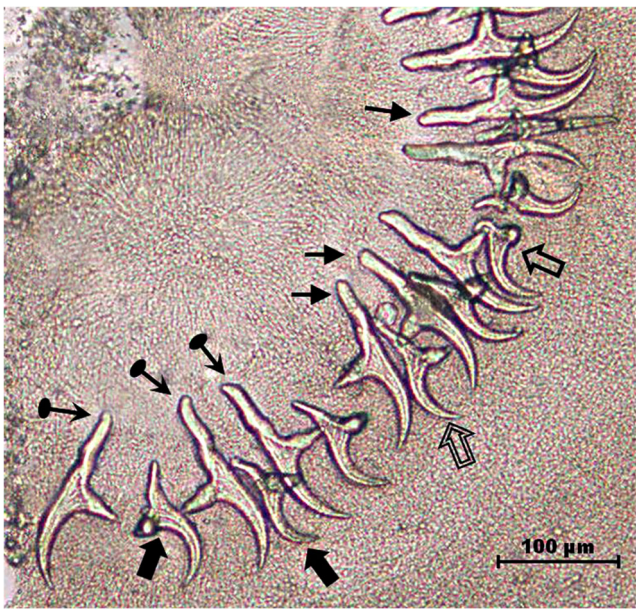

Fig. 2 Rostellar hooks of an adult worm from a cerebral coenurus cyst of sheep

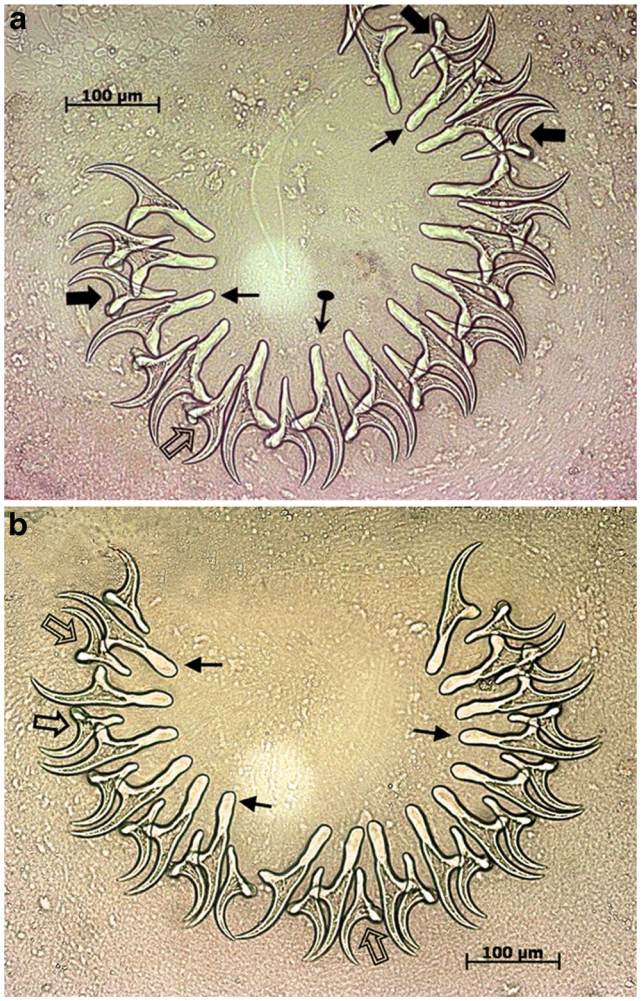

Fig. 3 Hooks of two rostella of adult worms from a non-cerebral coenurus cyst of goat

Adult worms and common features of mature proglottids

The following numbers of adult worms were recovered after the experimental infection: 33 cerebral worms/sheep, 28 cerebral worms/goat, 26 non-cerebral worms/sheep, and 30 non-cerebral worms/goat. The general morphology of the stained mature proglottids (Figs. 5 and 6) illustrated in Fig. 1 closely resembles the description by Hall (1920).

Particularities of the mature proglottids

The testes of the non-cerebral worms are always close to the ovary or even partly overlapped it (Fig. 6), but the testes in the cerebral worms are usually distinctly separated (Fig. 5). The

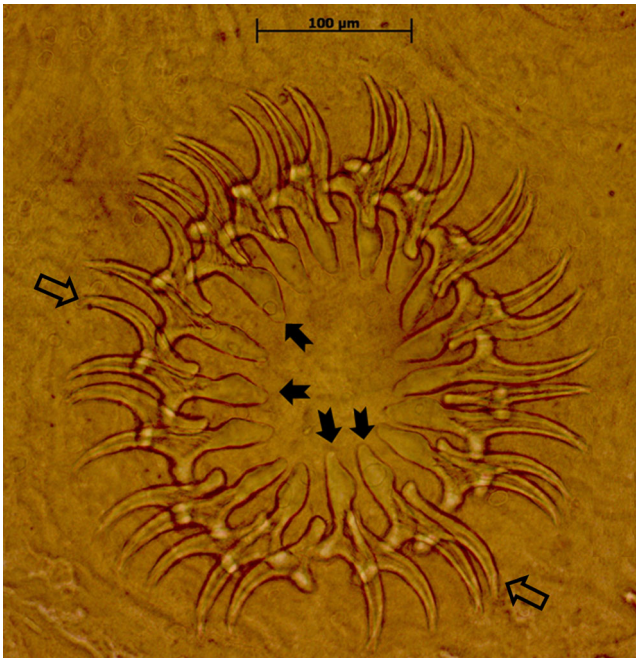

Fig. 4 Rostellar hooks of a protoscolex from a non-cerebral coenurus cyst of goat (*Coenurus gaigeri*). In Figs. 2, 3, and 4, the different arrows indicate: large hook handle ends bluntly, large hook handle ending with a slight curvature dorsally at the distal extremity, large hooks with a large notch on the dorsal border of their handles, small hook with a curved handle, small hooks with handles that turn dorsally at the distal extremity (in Fig. 2a, the small hook indicated by this arrow is described in the text as having a sigmoid appearance), small hooks with a straight handle

testes in some cases of cerebral worms, however, are also pressed closely to the ovary. The coils of the VD in non-cerebral worms are very tightly spiralled, and the VD has a nearly uniform width along its course (Figs. 6 and 7). The coils of the VD in cerebral worms are usually looser, and the VD varies in width along its course, forming a spindle shape (Figs. 5 and 8). The median top of the CP in cerebral worms is frequently curved towards the anterior portion of the segment (Fig. 8), but it can sometimes remain quite straight. The median top of the CP is usually straight in non-cerebral worms and is only rarely slightly curved, when the concavity is on the posterior side, towards the vagina (Fig. 7).

Statistical analyses of the morphometric measurements

A total of 48 morphometric parameters (for protoscolices in cysts and adult worms cumulatively) were analysed on the basis of cyst location (non-cerebral vs. cerebral) and host species (coenurus cerebralis/sheep, coenurus cerebralis/goat, coenurus skrjabini/sheep and coenurus gaigeri/goat). Thirty-six parameters differed significantly for both cyst location and host species, four differed significantly for only the cyst location, and three differed significantly for only the host species ($p < 0.05$). Five parameters did not differ significantly for these variables. For brevity, Tables 1, 2, 3, and 4 show the results for only a selection of the parameters analysed.

Molecular sequences and phylogenetic trees

All sequences for the cerebral and non-cerebral cases showed the highest degree of identity with sequences of *T. multiceps* from GenBank using the National Center for Biotechnology Information BLAST programmes and databases. In total, 6, 10, and 5 haplotypes were detected for the *nad*1, *cox*1, and 12S rRNA genes, respectively, but 2, 3, and 2 of these haplotypes, respectively, did not match 100 % with the sequences in GenBank (see Table 4). All haplotypes were deposited in GenBank under the accession numbers listed in Table 4.

Of the 6 *nad*1 haplotypes, one (N1) was found in both cerebral and non-cerebral specimens, one (N2) was found only in non-cerebral specimens, and four (N3, N4, N5, and N6) were found only in cerebral specimens. Of the 10 *cox*1 haplotypes, one (C1) was found in both cerebral and non-cerebral specimens, five (C2, C3, C4, C5, and C6) were found only in non-cerebral specimens, and four (C7, C8, C9, and C10) were found only in cerebral specimens. Of the 5 12S rRNA-gene haplotypes, two (S1 and S2) were found in both cerebral and non-cerebral specimens and three (S3, S4, and S5) were found only in non-cerebral specimens (Table 4).

Based on the concatenated sequences of the *nad*1, *cox*1, and 12S rRNA genes, 14 and 8 parasite haplotypes were identified among the 26 non-cerebral and the 26 cerebral specimens, respectively. None of the haplotypes were identical between the non-cerebral and cerebral cases. The sequence alignments indicated that 0.26 and 2.63 % of the *nad*1 positions (1/380 and 10/380), 2.96 and 2.66 % of the *cox*1 positions (10/338 and 9/338), and 1.44 and 0.36 % of the 12S rRNA positions (4/277 and 1/277) were polymorphic in the non-cerebral and cerebral isolates, respectively.

The phylogenetic relationships among *nad*1 haplotypes detected in this study and other *Taenia* isolates are presented in Fig. 9a (phylogenetic trees for *cox*1 and 12S rRNA-gene are not shown). A phylogramme of the concatenated sequences (*nad*1-*cox*1-12S rRNA) produced in this study is shown in Fig. 9b, and the parsimonious haplotype network based on the concatenated sequences is presented in Fig. 10.

Correlation between the molecular and morphological data

We found clear morphological differences between the cerebral parasitic stages and the non-cerebral parasitic stages, even for those clustering together in the phylogenetic analysis (Fig. 9a, b). For example, the Greek samples G1, G3, G10, G17, and G19 had characteristics (e.g. hooks and distribution of testes) of the cerebral parasitic stages, even though they

Table 1 Comparison of the morphological characteristics of the hooks of coenurus cysts and experimentally produced *Taenia* spp.

Measurement	Tissue affected and origin	Protoscolices in coenurus cysts				Experimentally produced *Taenia* spp.			
		N	Mean	SD	*p*	*N*	Mean	SD	*p*
Number of large hooks (same as small hooks)	Non-cerebral	90	14.71	1.16	0.237	27	14.63	0.69	0.001*
	Cerebral	80	14.40	1.45		27	15.37	0.84	
	C. gaigeri/goat	78	14.73 a	1.14	0.657	18	14.61 c	0.61	0.008**
	C. skrjabini/sheep	12	14.58 a	1.38		9	14.67 b c	0.87	
	C. cerebralis/sheep	63	14.43 a	1.50		18	15.28 a b	0.89	
	C. cerebralis/goat	17	14.29 a	1.38		9	15.56 a	0.73	
Total length of large hooks (μm)	Non-cerebral	516	152.64	11.12	0.000*	174	164.84	5.48	0.000*
	Cerebral	407	159.51	6.24		186	161.28	6.87	
	C. gaigeri/goat	438	152.46 b[a]	11.22	0.000**	130	163.97 a	5.61	0.000**
	C. skrjabini/sheep	78	153.69 b	10.50		44	167.41 b	4.19	
	C. cerebralis/sheep	312	159.68 a	5.76		128	161.17 c	6.29	
	C. cerebralis/goat	95	158.83 a	7.98		58	161.52 c	8.05	
Length of handle of large hooks (μm)	Non-cerebral	516	78.27	8.95	0.037*	174	86.26	5.13	0.000*
	Cerebral	407	79.74	4.82		186	80.25	5.79	
	C. gaigeri/goat	438	78.33 b	8.95	0.189	130	85.44 b	5.21	0.000**
	C. skrjabini/sheep	78	77.92 a b	8.10		44	88.70 a	4.04	
	C. cerebralis/sheep	312	79.90 a	4.64		128	80.44 c	5.48	
	C. cerebralis/goat	95	79.09 a	5.50		58	79.81 c	6.44	
Length of blade of large hooks (μm)	Non-cerebral	516	74.27	5.49	0.000*	174	75.25	4.21	0.000*
	Cerebral	407	78.61	3.77		186	79.17	2.89	
	C. gaigeri/goat	438	73.97 c	5.47	0.000**	130	75.33 b	4.55	0.000**
	C. skrjabini/sheep	78	75.94 b	5.36		44	75.00 b	2.98	
	C. cerebralis/sheep	312	78.45 a	3.51		128	79.05 a	3.01	
	C. cerebralis/goat	95	79.23 a	4.72		58	79.43 a	2.60	
Total length of small hooks (μm)	Non-cerebral	500	111.84	8.57	0.000*	167	116.96	8.27	0.000*
	Cerebral	439	107.96	7.76		161	106.57	7.24	
	C. gaigeri/goat	429	111.59 a	8.62	0.000**	120	116.88 a	8.18	0.000**
	C. skrjabini/sheep	71	113.38 a	8.14		47	117.17 a	8.58	
	C. cerebralis/sheep	308	108.12 b	7.63		105	106.74 b	5.86	
	C. cerebralis/goat	131	107.39 b	8.33		56	106.25 b	9.35	
Length of handle of small hooks (μm)	Non-cerebral	500	53.40	7.36	0.000*	167	55.55	7.37	0.000*
	Cerebral	439	46.53	6.92		161	46.77	5.68	
	C. gaigeri/goat	429	53.21 a	7.49	0.000**	120	55.51 a	7.07	0.000**
	C. skrjabini/sheep	71	54.61 a	6.44		47	55.66 a	8.18	
	C. cerebralis/sheep	308	46.69 b	6.82		105	47.46 b	5.38	
	C. cerebralis/goat	131	45.97 b	7.38		56	45.48 b	6.04	
Length of blade of small hooks (μm)	Non-cerebral	500	58.05	3.67	0.001*	167	57.61	2.16	0.799
	Cerebral	439	59.19	2.84		161	57.53	3.21	
	C. gaigeri/goat	429	57.92 b	3.70	0.001**	120	57.52 a b	2.23	0.181
	C. skrjabini/sheep	71	58.86 a	3.44		47	57.85 a b	1.97	
	C. cerebralis/sheep	308	59.07 a	2.76		105	57.21 b	3.09	
	C. cerebralis/goat	131	59.61 a	3.08		56	58.14 a	3.36	

The comparison was based on tissue affected (non-cerebral vs. cerebral) and parasite source (*C. gaigeri*/goat vs. *C. skrjabini*/sheep vs. *C. cerebralis*/sheep vs. *C. cerebralis*/goat)

*significant difference between the measurements in the same column on the basis of tissue affected ($p < 0.05$); **significant difference between the measurements in the same column on the basis of parasite source ($p < 0.05$)

[a] Means for the same measurement that do not share a letter are significantly different

clustered with the tropical/subtropical samples in Figs. 9b and 10 and had the *nad*1 N1 and *cox*1 C1 haplotypes, i.e. haplotypes common with the tropical/subtropical samples (Table 4). Likewise, no noteworthy morphological differences

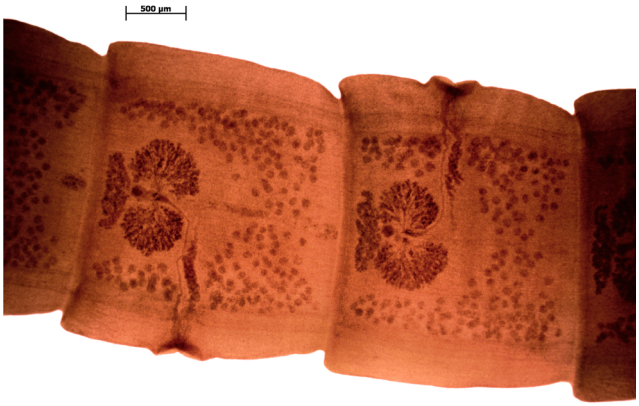

Fig. 5 Mature proglottis of an adult worm from a cerebral coenurus cyst of sheep

were detected between the Greek samples grouping in different clades or between the tropical/subtropical samples that also belonged to different clades of Fig. 9a, b.

Discussion

In contrast to previous studies, the non-cerebral cysts in this study originated from a wide range of geographical regions in the tropics (from Bangladesh to Ethiopia) and from different host species (sheep and goats) and are compared with cerebral cysts from the extended area of continental Greece, where cerebral coenurosis is common (Christodoulopoulos et al. 2008) but where non-cerebral coenurosis has never been described to the best of our knowledge.

The referred origin of the samples of non-cerebral coenurosis was based on information obtained from animal traders in conjunction with the breed of the animals. The credibility of the exact origin for each sample is thus questionable, and the origin data are reported here with caution. In contrast, we can confirm that the affected animals with cerebral coenurosis had been born and reared in the Greek county stated in this paper.

Fig. 6 Mature proglottis of an adult worm from a non-cerebral coenurus cyst of goat

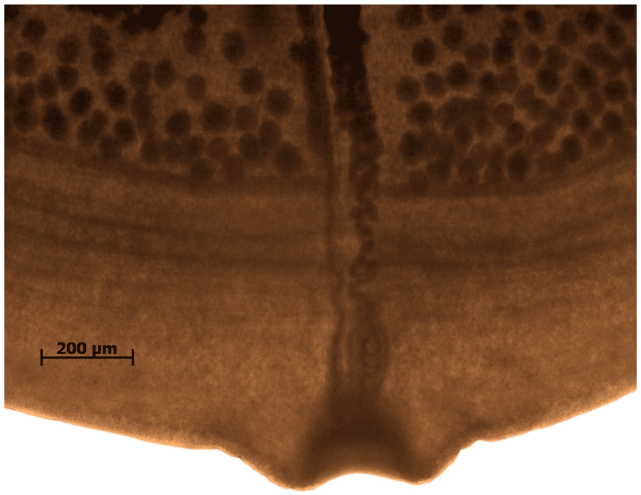

Fig. 7 Details of the male reproductive organs of an adult worm from a non-cerebral coenurus cyst of sheep

Rostami et al. (2013) reported a statistically significant difference in hook length among individual isolates of *T. multiceps*. The proglottis morphology of adult *T. multiceps* and the formerly named *T. gaigeri* have only been described by Hall (1920). The work of Verster (1969) was important but was limited to reporting the total number of anatomical features, such as the testes, and did not address their distribution in the body of the parasites, as in the present study. Also, both Hall (1920) and Verster (1969) examined only one specimen of *T. gaigeri*, an adult worm derived from a non-cerebral coenurus cyst of a goat.

Akbari et al. (2015) recently described the experimental reproduction of adult worms from protoscolices of coenurus cysts collected from the brains of sheep and the muscles of goats from the same geographical area of southern Iran. The authors concluded that the same species produced both cerebral

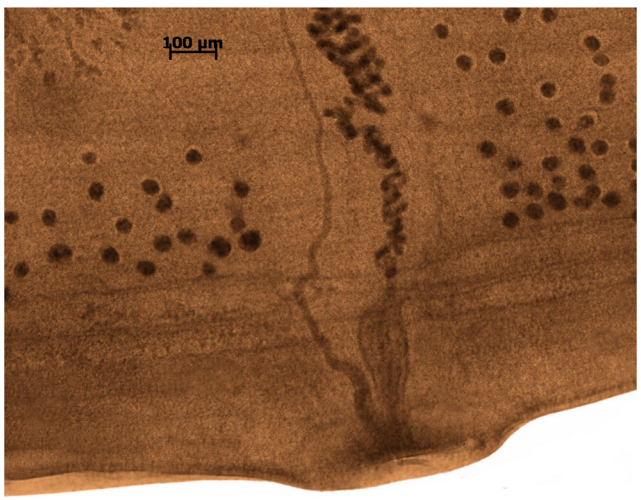

Fig. 8 Details of the male reproductive organs of an adult worm from a cerebral coenurus cyst of sheep

Table 2 Comparison of the morphological characteristics of the testes of mature proglottids of experimentally produced *Taenia* spp.

Measurement	Tissue affected and parasite source	*N*	Mean[a]	SD	*p*
Number of testes in poral side of mature proglottids posterior to the vagina	Non-cerebral	108	52.22	4.81	0.000*
	Cerebral	108	31.18	5.40	
	C. gaigeri/goat	72	52.83 a	4.63	0.000**
	C. skrjabini/sheep	36	51.00 a	5.00	
	C. cerebralis/sheep	72	32.22 b	5.27	
	C. cerebralis/goat	36	29.11 c	5.10	
Number of testes in poral side of mature proglottids anterior to the vas deferens	Non-cerebral	108	87.35	8.34	0.000*
	Cerebral	108	109.12	15.59	
	C. gaigeri/goat	72	87.76 c	8.49	0.000**
	C. skrjabini/sheep	36	86.53 c	8.08	
	C. cerebralis/sheep	72	111.06 a	16.16	
	C. cerebralis/goat	36	105.25 b	13.78	
Number of testes in poral side of mature proglottids	Non-cerebral	108	139.57	11.63	0.712
	Cerebral	108	140.31	17.00	
	C. gaigeri/goat	72	140.60 a b	11.43	0.016**
	C. skrjabini/sheep	36	137.53 b c	11.90	
	C. cerebralis/sheep	72	143.28 a	17.44	
	C. cerebralis/goat	36	134.36 c	14.58	
Number of testes in non-poral side of mature proglottids	Non-cerebral	108	149.56	13.32	0.086
	Cerebral	108	146.39	13.67	
	C. gaigeri/goat	72	87.76 c	8.49	0.000**
	C. skrjabini/sheep	36	86.53 c	8.08	
	C. cerebralis/sheep	72	111.06 a	16.16	
	C. cerebralis/goat	36	105.25 b	13.78	
Number of testes in mature proglottids	Non-cerebral	108	289.13	22.48	0.468
	Cerebral	108	286.69	26.56	
	C. gaigeri/goat	72	291.18 a	19.68	0.028**
	C. skrjabini/sheep	36	285.03 a b	27.07	
	C. cerebralis/sheep	72	291.11 a	28.62	
	C. cerebralis/goat	36	277.86 b	19.35	

The comparison was based the tissue affected (non-cerebral vs. cerebral) and parasite source (*C. gaigeri*/goat vs. *C. skrjabini*/sheep vs. *C. cerebralis*/sheep vs. *C. cerebralis*/goat)

*significant difference between the measurements on the basis of tissue affected ($p < 0.05$); **significant difference between the measurements on the basis of parasite source ($p < 0.05$)

[a] Means for the same measurement that do not share a letter are significantly different

and non-cerebral cysts in sheep and goats of the above area. This conclusion was in support of previous data from Iran, where the forms causing non-cerebral coenurosis could also affect the brain to produce cerebral cases (Kheirandish et al. 2012).

Our results for a number of morphometric features disagreed with those by Akbari et al. (2015), e.g. Akbari et al. (2015) reported 135.6 ± 6.3 testes in the mature proglottids of the adult taenias originating from the non-cerebral coenuri, but we found 289.13 ± 22.48. Our results, however, agreed with many of the differences between *T. gaigeri* and *T. multiceps* described by Hall (1920), especially those for the morphology of the hooks. We disagree with two of the differences stated by Hall (1920) in his key to species of *Taenia*. Our data indicated that the handles of the large hooks did not always taper in the cerebral parasitic stages, which was also found in the large hooks of some non-cerebral forms, albeit much more rarely. In addition, our data supported the extension of the testes posterior to the ovaries to a greater degree in non-cerebral worms than in cerebral worms, but we did not find any testes between the vitellarium and the ovaries in any of the examined worms (Figs. 5 and 6).

Our study found several characteristic differences of the adult proglottids between cerebral and non-cerebral worms (Table 3; Figs. 5, 6, 7, and 8). The difference in the distribution of the testes on the poral side of the proglottids was a particularly interesting observation. Cerebral worms had approximately threefold more testes in front of the VD than between the ovary and the vagina, whereas non-cerebral worms had

Table 3 Comparison of the morphological characteristics of mature proglottids of experimentally produced *Taenia* spp.

Measurement	Tissue affected and parasite source	*N*	Mean[a]	SD	*p*
Length of cirrus pouch of mature proglottids (mm)	Non-cerebral	108	0.26	0.04	0.000*
	Cerebral	108	0.34	0.05	
	C. gaigeri/goat	72	0.26 b	0.04	0.000**
	C. skrjabini/sheep	36	0.27 b	0.04	
	C. cerebralis/sheep	72	0.34 a	0.05	
	C. cerebralis/goat	36	0.34 a	0.05	
Max width of cirrus pouch of mature proglottids (mm)	Non-cerebral	108	0.10	0.02	0.000*
	Cerebral	108	0.12	0.03	
	C. gaigeri/goat	72	0.10 c	0.02	0.000**
	C. skrjabini/sheep	36	0.10 c	0.02	
	C. cerebralis/sheep	72	0.12 b	0.03	
	C. cerebralis/goat	36	0.13 a	0.03	
Length of vas deferens of mature proglottids (mm)	Non-cerebral	108	0.75	0.09	0.000*
	Cerebral	108	0.63	0.11	
	C. gaigeri/goat	72	0.75 a	0.07	0.000*
	C. skrjabini/sheep	36	0.75 a	0.12	
	C. cerebralis/sheep	72	0.63 b	0.12	
	C. cerebralis/goat	36	0.64 b	0.10	
Distance between genital atrium and lateral nerve of mature proglottids (mm)	Non-cerebral	108	0.25	0.03	0.003*
	Cerebral	108	0.23	0.04	
	C. gaigeri/goat	72	0.25 a	0.03	0.029**
	C. skrjabini/sheep	36	0.25 a	0.05	
	C. cerebralis/sheep	72	0.23 b	0.04	
	C. cerebralis/goat	36	0.23 a b	0.05	
Distance between genital atrium and lateral side of the longitudinal excretory canal of mature proglottids (mm)	Non-cerebral	108	0.32	0.04	0.000*
	Cerebral	108	0.29	0.05	
	C. gaigeri/goat	72	0.32 a	0.03	0.000**
	C. skrjabini/sheep	36	0.32 a	0.05	
	C. cerebralis/sheep	72	0.28 b	0.04	
	C. cerebralis/goat	36	0.29 b	0.06	
Distance between genital atrium and medial side of the longitudinal excretory canal of mature proglottids (mm)	Non-cerebral	108	0.40	0.04	0.047*
	Cerebral	108	0.38	0.06	
	C. gaigeri/goat	72	0.40 a	0.03	0.197
	C. skrjabini/sheep	36	0.39 a b	0.06	
	C. cerebralis/sheep	72	0.38 b	0.05	
	C. cerebralis/goat	36	0.38 a b	0.07	

The comparison was based on the tissue affected (non-cerebral vs. cerebral) and parasite source (*C. gaigeri*/goat vs. *C. skrjabini*/sheep vs. *C. cerebralis*/sheep vs. *C. cerebralis*/goat)

*significant difference between the measurements on the basis of tissue affected ($p < 0.05$); **significant difference between the measurements on the basis of parasite source ($p < 0.05$)

[a] Means for the same measurement that do not share a letter are significantly different

less than twofold more testes in front of the VD than between the ovary and vagina (Table 2).

The interpretation of the significant differences noted in Tables 1, 2, and 3 along with the above observations led to the following conclusions: (a) non-cerebral parasitic stages (adult worms and coenuri)/sheep do not significantly differ morphologically from non-cerebral parasitic stages/goat nor do cerebral parasitic stages/sheep differ from cerebral parasitic stages/goat. (b) The cerebral parasitic stages and the non-cerebral stages have distinct morphological differences.

The question that emerges is whether the variants that cause the non-cerebral coenurosis should be considered as a species different from *T. multiceps*. Addressing this question from a morphological aspect is a complex task. Species of the genus *Taenia* are not easily identified morphologically, because many of the characters overlap (Loos-Frank 2000). The classical view of systematic parasitology is that a standard and invariable morphological feature, such as the third ovarial lobe in *T. solium*, may justify the creation of a new species (Verster 1969). Nevertheless, we did not find a distinct morphological feature that unequivocally differed between non-cerebral worms and cerebral worms. The morphological differences described here between these two investigated groups instead fall within the variation of *T. multiceps* (Hall 1920; Loos-Frank 2000).

Our molecular phylogenetic analysis, however, indicates that the parasites that cause non-cerebral coenurosis in sheep

Table 4 Haplotypes of coenurus cysts from cerebral and non-cerebral cases

Gene	Haplotype	GenBank accession no.	Highest GenBank identity	Non-cerebral cases		Cerebral cases	
				Samples	Host	Samples	Host
*nad*1	N1	KX505144	100 % (e.g. KC794809)	T2, T4, T5, T6, T8, T12, T16, T19, T21, T23	Goat	G1, G3, G10, G17, G19	Sheep
				T10, T11, T25	Sheep		
	N2	KX505145	100 % (e.g. HM101470)	T1, T3, T7, T9, T13, T14, T15, T17, T18, T20, T22	Goat		
				T24, T26	Sheep		
	N3	KX505146	100 % (e.g. FJ495086)			G4, G6, G8, G9, G11, G14, G15, G20	Sheep
						G25	Goat
	N4	KX505147	100 % (e.g. GQ228818)			G5, G7, G12, G13, G16	Sheep
						G21, G22, G23, G24, G26	Goat
	N5	KX505148	99 % (e.g. DQ077820)			G2	Sheep
	N6	KX505149	99 % (e.g. KR604805)			G18	Sheep
*cox*1	C1	KX505150	100 % (e.g. KX522563)	T10	Sheep	G1, G3, G10, G17, G19	Sheep
	C2	KX505151	100 % (e.g. HM101469)	T1, T2, T4, T5, T6, T7, T9, T13, T19, T20, T22	Goat		
				T24, T25	Sheep		
	C3	KX505152	99 % (e.g. JX507239)	T12, T14, T15, T16, T17, T18, T23	Goat		
				T26	Sheep		
	C4	KX505153	99 % (e.g. JX507220)	T3, T21	Goat		
	C5	KX505154	99 % (e.g. HM101469)	T8	Goat		
	C6	KX505155	99 % (e.g. DQ309767)	T11	Sheep		
	C7	KX505156	100 % (e.g. FJ495086)			G4, G6, G8, G9, G11, G12, G14, G15, G18, G20,	Sheep
						G23, G25	Goat
	C8	KX505157	100 % (e.g. KR604807)			G5, G7, G16	Sheep
						G21, G22, G24, G26	Goat
	C9	KX505158	100 % (e.g. GQ228818)			G2	Sheep
	C10	KX505159	100 % (e.g. JQ710587)			G13	Sheep
12S rRNA	S1	KX505160	100 % (e.g. GQ228818)	T10, T11, T25	Sheep	G2, G4, G5, G6, G7, G8, G9, G11, G12, G13, G14, G15, G16, G18, G20	Sheep
						G21, G22, G23, G24, G25	Goat
	S2	KX505161	100 % (e.g. JQ710631)	T1, T3, T8, T9, T12, T13, T14, T16, T17, T18, T19, T20, T21, T22, T23	Goat	G1, G3, G10, G17, G19	Sheep
				T24	Sheep	G26	Goat
	S3	KX505162	100 % (e.g. KX377709)	T2, T4, T6	Goat		
	S4	KX505163	99 % (e.g. FJ495086)	T5	Goat		
	S5	KX505164	99 % (e.g. GQ228818)	T7, T15	Goat		
				T26	Sheep		

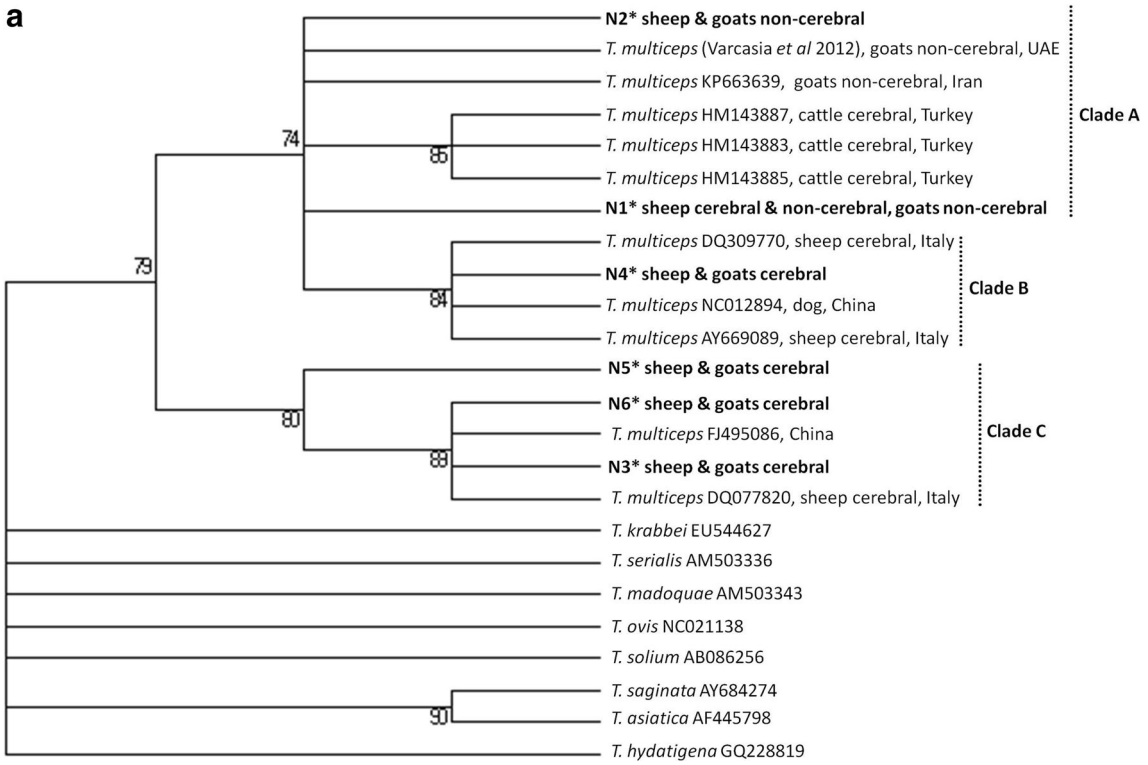

Fig. 9 Maximum-likelihood phylogenetic trees based on the *nad*1 (**a**) and the concatenated *nad*1-*cox*1-12S rRNA sequences (**b**) (model HKY+G; 1000 bootstraps; cutoff 70 %). The *asterisk* in **a** indicates the haplotypes of our specimens (see Table 4). The concatenated haplotype code is given in **b** for each listed isolate

and goats appear to cluster with other isolates of *T. multiceps* (Fig. 9a, b). This conclusion had already been proposed in previous molecular studies of the causative parasite of non-cerebral coenurosis in goats (Oryan et al. 2010; Varcasia et al. 2012; Akbari et al. 2015; Amrabadi et al. 2015).

In our study, the phylogenetic trees of *nad*1 and concatenated sequences provided the best information for differentiating and identifying the relationships between the isolates (Fig. 9a), and the *cox*1 and 12S rRNA tree was less informative (trees do not show). The phylogenetic analysis of the concatenated sequences and those of *nad*1 indicated that no monophyletic groups were based on geographical origin, organ location in the intermediate host (cerebral or non-cerebral), or species of intermediate host (sheep or goat). Our molecular analysis thus indicates that the variants causing non-cerebral coenurosis should not be considered as a species different from *T. multiceps*. The analysis, however, suggested that a haplotypic cluster (clade A) was able to induce both cerebral and non-cerebral cases and that two additional clusters (clades B and C) had a predilection only for the brain (Fig. 9a, b).

Additionally, our isolates from non-cerebral cases tended to cluster with GenBank isolates from both non-cerebral and cerebral cases in Iran and Turkey, and our isolates from cerebral cases clustered with GenBank isolates from cerebral cases in Italy and China (Fig. 9a). The tendency for the polarisation of our non-cerebral isolates relative to our cerebral isolates and other cerebral isolates from GenBank is obvious in the phylogenetic tree of the concatenated sequences (Fig. 9b), especially in the parsimonious networks (Fig. 10). The parsimonious networks also tended to cluster based on the species of the intermediate host (sheep or goat) (Fig. 10). The clustering based on geographical origin (North hemisphere vs. tropics and subtropics) in the overall analysis, however, was the most noteworthy (Figs. 9a, b and 10).

The clustering of the non-cerebral parasitic forms in the present molecular analysis is indicative of intraspecific variation, which has been illustrated in *T. multiceps* in various studies (Varcasia et al. 2006; Avcioglu et al. 2011; Rostami et al. 2013). Rostami et al. (2013) has associated the high genetic diversity of *T. multiceps* with the ability, as with *Echinococcus* spp., of a single oncosphere to give rise to several hundred protoscolices in the form of a coenurus cyst. A single mutant oncosphere can therefore produce many genetically identical individuals potentially capable of generating new genetic variants in the life cycle of the parasite. The large intraspecific variation within *E. granulosus sensu lato* led to the designation of several new species, e.g. *E. granulosus sensu stricto* (G1–G3 genotypes), *E. equinus* (G4), *E. ortleppi* (G5), and *E. canadensis* (G6–G10) (Thompson 2008). Intraspecific variation, however, is also

Fig. 9 (continued)

high in *Hydatigera taeniaeformis*, which does not asexually propagate in the intermediate host. The presence of cryptic species within *H. taeniaeformis* is commonly acknowledged (Nakao et al. 2013), but their taxonomic status remains unresolved due to limited morphological, molecular, biogeographical, and ecological data (Lavikainen et al. 2016).

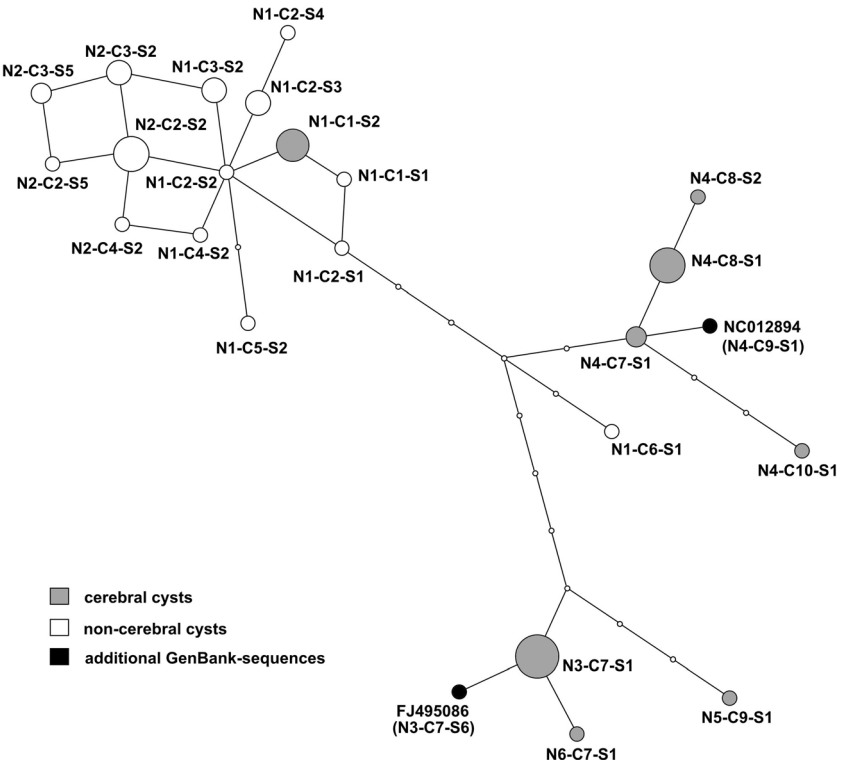

Fig. 10 Parsimonious network analysis based on the haplotypes of the concatenated *nad*1-*cox*1-12S rRNA sequences. The *size of the circles* indicates the frequency of the haplotypes (also see Fig. 9b). S6 is the deposited haplotype for the gene encoding 12S rRNA from the isolate FJ495086 (not present in our isolates)

Several studies have attributed the variability of *T. multiceps* with either the diversity of the sheep, goat, and cattle hosts (Varcasia et al. 2006, 2012) or the geographical range of the samples (Rostami et al. 2013; Akbari et al. 2015). Verster (1969) associated the cerebral and non-cerebral parasitic forms of *T. multiceps* with the species of the intermediate host: coenuri mature only in nervous tissue in sheep but may reach maturity in other organs in goats.

The results of this study support the association of *T. multiceps* variability with the geographical origin of the isolates and lead us to propose a reformulated hypothesis for the existence of cerebral and non-cerebral forms. Genetic distances within our set of isolates suggest that the development of cerebral coenuri in sheep may be an ancestral property of *T. multiceps*; all variants are therefore able to produce cerebral forms, but only some variants have acquired the additional capacity to affect the brain of other species (goats and cattle) or to produce non-cerebral forms, mostly in goats and more rarely in sheep. These conclusions are supported by the basal position of the cerebral specimens in the tree in Fig. 9b and by the lower diversity in the non-cerebral samples from an extended area of the tropics/subtropics compared to the diversity in the cerebral samples collected only from Greece (Table 4). The apparent absence of non-cerebral forms from some areas (e.g. Greece) and their regular occurrence, e.g. in the East and the tropics, calls for further investigation of the biogeography of this parasite and possible adaptive advantages of this phenotypic variant.

The above hypothesis is consistent with the results of all recent studies. Rostami et al. (2013) reported that all *T. multiceps* isolates from cerebral cases in Iranian sheep clustered in the same clade with isolates from goat non-cerebral cases originating from Iran and the UAE. Varcasia et al. (2013) described the molecular homology of bovine cerebral coenurosis in Sardinia with a specific strain of *T. multiceps* that had been previously described as the cause of sheep cerebral coenurosis in the same area (Scala and Varcasia 2006). Akbari et al. (2015) found that the same parasite produced both cerebral and non-cerebral forms in sheep and goats in southern Iran. Amrabadi et al. (2015) found that the cerebral cysts in sheep in Iran were 100 % genetically identical to the non-cerebral cysts in goats.

The genetic clusters in Fig. 9a, b were not correlated with morphological parameters other than those mentioned between non-cerebral and cerebral parasitic stages. The biological interpretation of this lack of correlation is not known. Morphological changes may be caused by genetic differences that are not detected with the markers used (e.g. in the nuclear genome) or by the induction of developmental alterations by the organ containing the coenuri.

Our study, however, clearly indicates a molecular basis for the non-cerebral pathogenicity in certain variants of *T. multiceps*. Specific biosecurity actions should therefore be enforced in areas without non-cerebral coenurosis and focused to prevent the introduction of *T. multiceps* variants with such pathogenicity.

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The graph of the proglottid (Fig. 1) has been adapted from a pattern of a stained mature proglottid of a taenia derived from a non-cerebral coenurus cyst of a goat. The graph was made by the artist Eunomia Dimitriadi, who is also a veterinarian. The authors would like to express their thanks to Eunomia for this prestigious artwork.

Compliance with ethical standards All investigations complied with the current laws of the countries in which they were performed. All animals were handled by trained and experienced veterinary staff following the recommendations of European Council Directive 86/609/EC for the protection of animals used for experimental purposes.

Conflict of interest The authors declare that they have no conflicts of interest.

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Supplement to paper 2.3.

Fig. S1. Diagram of mature proglottid of adult worm from non-cerebral coenurus cysts of goats. **S1a:** Shaded area indicates area of proglottids that is measured. **S1b:** Shaded area indicates area of proglottids surrounded by the excretory canals that is measured.

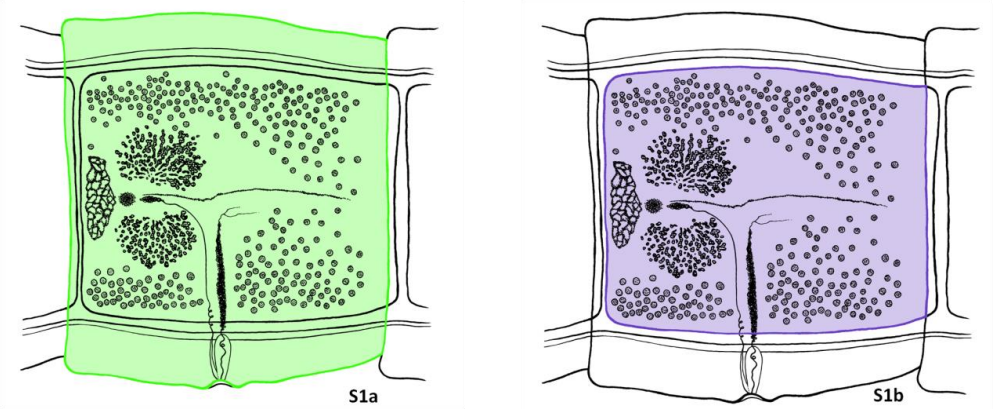

Fig. S2. Hooks of the rostellum of an adult worm from non-cerebral coenurus cyst of goat.

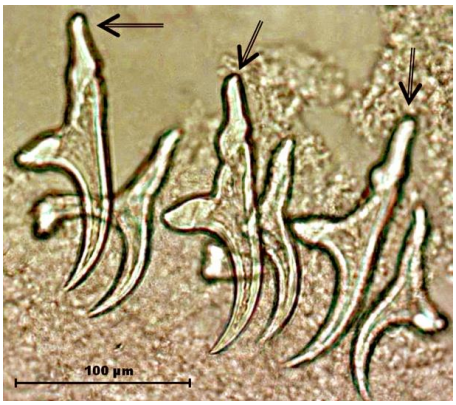

Fig. S3. Hooks of various rostella of adult worms from cerebral coenurus cyst of sheep.

In Figs S2 and S3, the different arrows indicate: → large hook handle ends bluntly, ⟶ large hook handle ending with a slight curvature dorsally at the distal extremity, ⟹ large hook handle tapering toward the distal extremity, ➡ small hook with curved handle, ⇨ small hooks with handles that turn dorsally at the distal extremity, ▷ small hooks with guard that appears of being bifid.

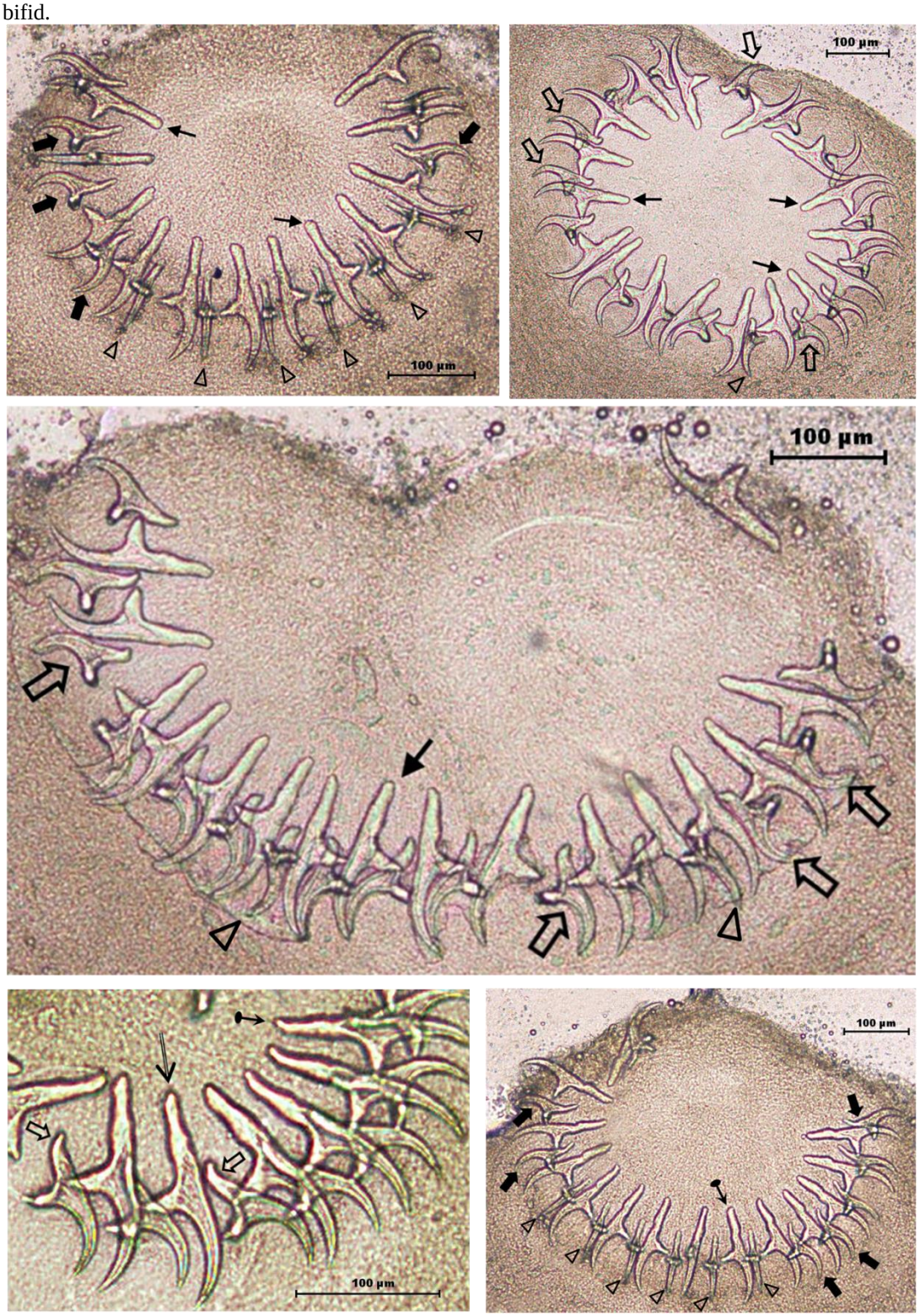

Fig S4. Mature proglottids of adult worm from cerebral coenurus cysts of sheep, with the testes press close to the ovary.

Fig S5. Proglottid with full development of the uterus and after the total disappearance of the ovary (gravid proglottid) of adult worm from cerebral coenurus cysts of sheep.

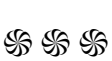

Fig. S6. Proglottid with full development of the uterus and after the total disappearance of the ovary (gravid proglottid) of adult worm from non-cerebral coenurus cysts of goats.

Fig. S7. Maximum-likelihood phylogenetic trees based on the *cox*1 sequences (model HKY+G; 1000 bootstraps; cut-off 70%). The asterisk indicates the haplotypes of our specimens (see Table 4 of the publication).

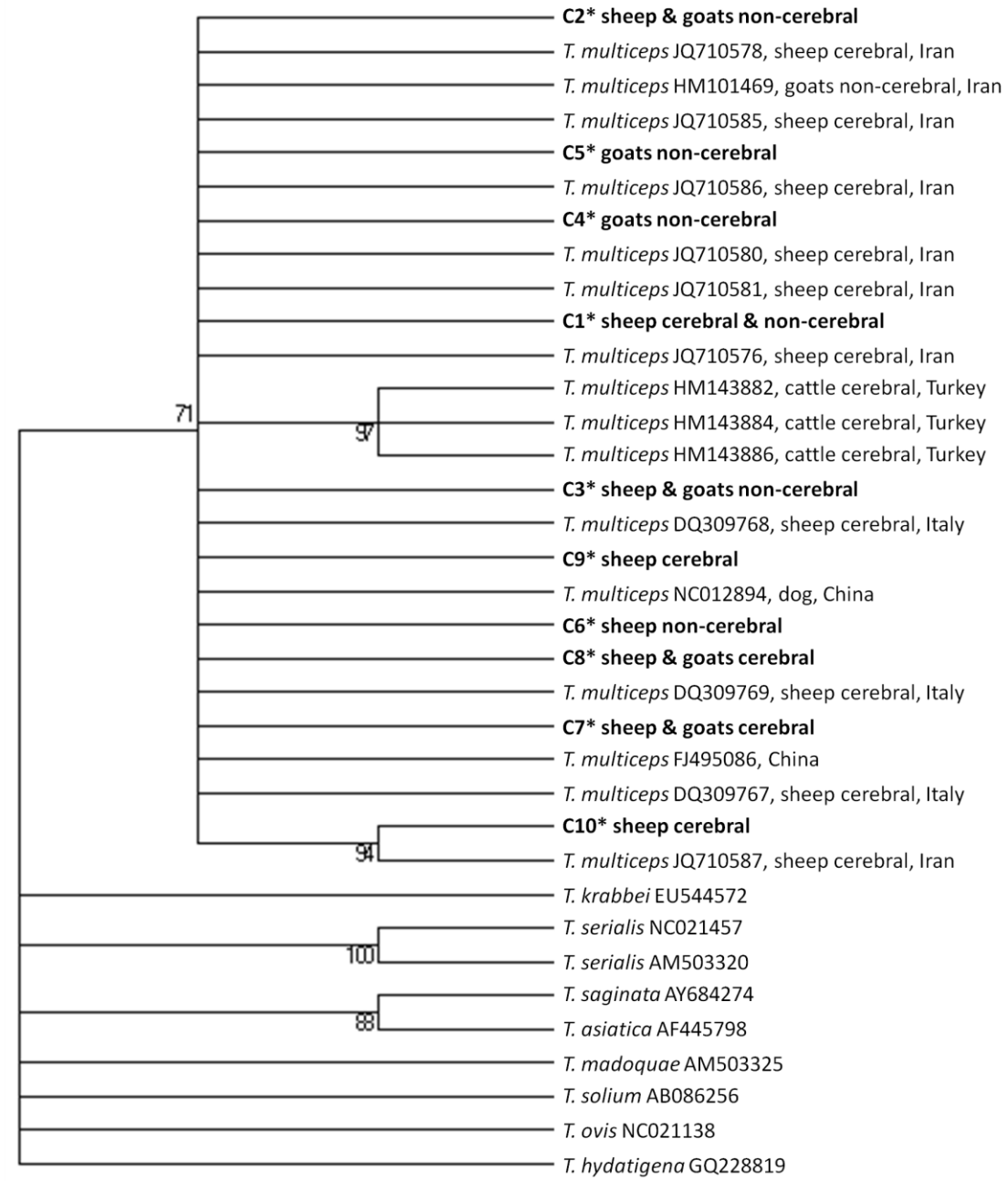

Fig. S8. Maximum-likelihood phylogenetic trees based on the 12S rRNA-gene sequences (model HKY+G; 1000 bootstraps; cut-off 70%). The asterisk indicates the haplotypes of our specimens (see Table 4 of the publication).

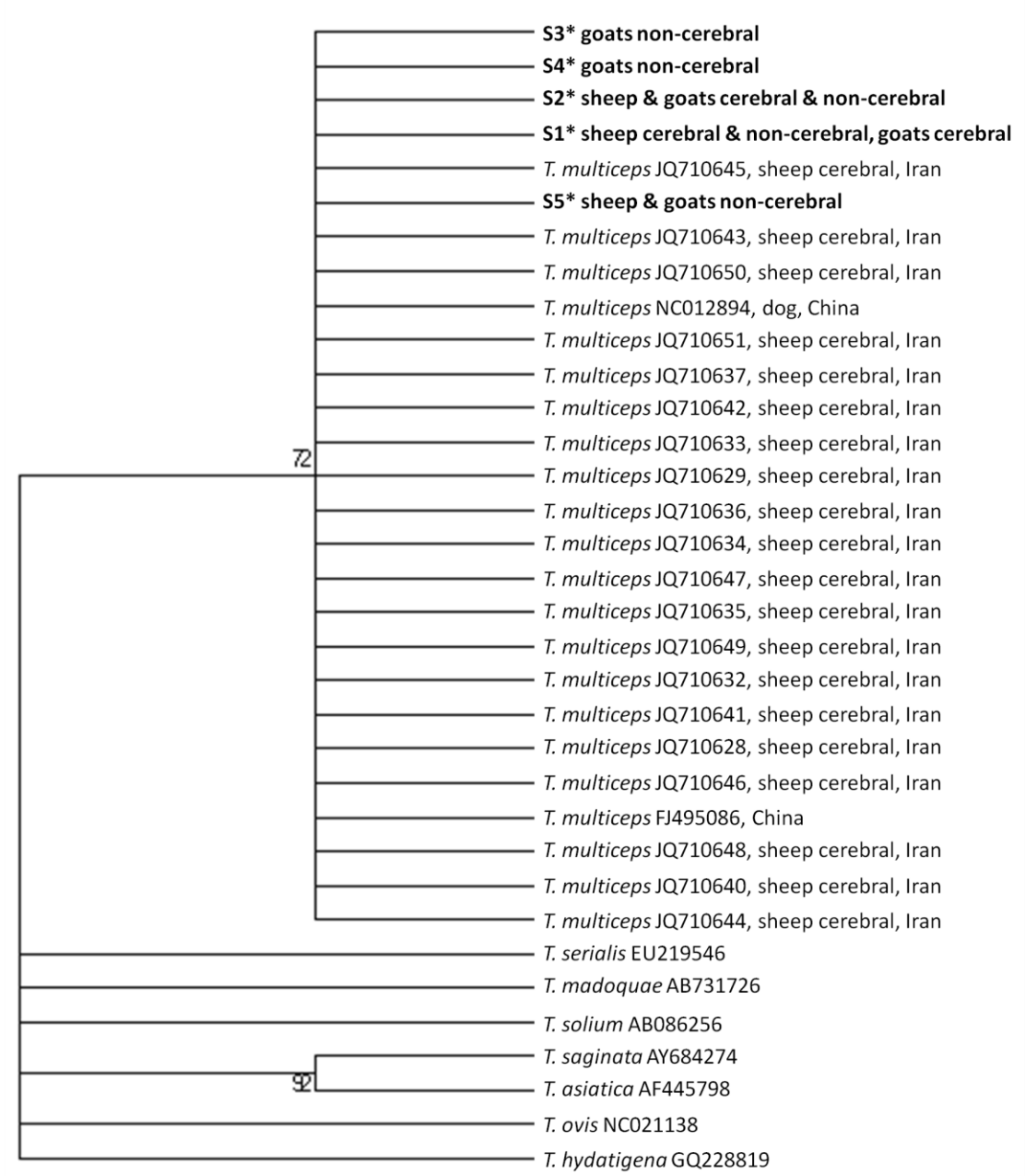

Table Si. Measurements not shown in Table 2.

Measurement	Tissue affected and parasite source	N	Mean[1]	Std	*P*
Ratio of testicles in poral side posterior of the vagina to testicles in poral side anterior of the vas deferens in mature proglottids	"Non-cerebral"	108	0.60	0.06	0.000*
	"Cerebral"	108	0.29	0.06	
	C. gaigeri/Goat	72	0.60 a	0.06	0.000**
	C. skrjabini/Sheep	36	0.59 a	0.05	
	C. cerebralis/Sheep	72	0.29 b	0.06	
	C. cerebralis/Goat	36	0.28 b	0.07	
Ratio of testicles in poral side to the total number of testicles in mature proglottids	"Non-cerebral"	108	0.48	0.02	0.068
	"Cerebral"	108	0.49	0.03	
	C. gaigeri/Goat	72	0.48 b	0.02	0.078
	C. skrjabini/Sheep	36	0.48 a b	0.02	
	C. cerebralis/Sheep	72	0.49 a	0.03	
	C. cerebralis/Goat	36	0.48 a b	0.03	
Ratio of testicles in poral side to the number of testicles in non-poral side in mature proglottids	"Non-cerebral"	108	0.93	0.07	0.038*
	"Cerebral"	108	0.96	0.10	
	C. gaigeri/Goat	72	0.93 b	0.07	0.054
	C. skrjabini/Sheep	36	0.94 a b	0.06	
	C. cerebralis/Sheep	72	0.97 a	0.10	
	C. cerebralis/Goat	36	0.94 a b	0.11	

The comparison was based the tissue affected ("Non-cerebral" vs. "Cerebral") and parasite source (*C. gaigeri*/Goat vs. *C. skrjabini*/Sheep vs. *C. cerebralis*/Sheep vs. *C. cerebralis*/Goat).

* Significant difference between the measurements on the basis of tissue affected ($P<0.05$).

** Significant difference between the measurements on the basis of parasite source ($P<0.05$).

[1] Different letters indicate significant differences.

Table Sii. Measurements not shown in Table 3.

Measurement	Tissue affected and parasite source	N	Mean[1]	Std	*P*
Area of mature proglottids (mm^2)	"Non-cerebral"	108	6.13	1.27	0.388
	"Cerebral"	108	6.33	1.97	
	C. gaigeri/Goat	72	6.12 a	1.04	0.863
	C. skrjabini/Sheep	36	6.14 a	1.66	
	C. cerebralis/Sheep	72	6.32 a	1.97	
	C. cerebralis/Goat	36	6.33 a	1.98	
Area through excretory canals of mature proglottids (mm^2)	"Non-cerebral"	108	3.83	0.83	0.008*
	"Cerebral"	108	4.28	1.54	
	C. gaigeri/Goat	72	3.81 a	0.64	0.072
	C. skrjabini/Sheep	36	3.87 a	1.14	
	C. cerebralis/Sheep	72	4.28 a	1.57	
	C. cerebralis/Goat	36	4.28 a	1.51	
Area of poral ovary lobe of mature proglottids (mm^2)	"Non-cerebral"	108	0.21	0.04	0.000*
	"Cerebral"	108	0.17	0.05	
	C. gaigeri/Goat	72	0.21 a	0.03	0.000**
	C. skrjabini/Sheep	36	0.21 a	0.05	
	C. cerebralis/Sheep	72	0.17 b	0.05	
	C. cerebralis/Goat	36	0.17 b	0.05	
Area of non-poral ovary lobe of mature proglottids (mm^2)	"Non-cerebral"	108	0.23	0.05	0.055
	"Cerebral"	108	0.21	0.07	
	C. gaigeri/Goat	72	0.22 a	0.03	0.270
	C. skrjabini/Sheep	36	0.23 a	0.07	
	C. cerebralis/Sheep	72	0.21 a	0.07	
	C. cerebralis/Goat	36	0.21 a	0.07	
Area of vitellarium of mature proglottids (mm^2)	"Non-cerebral"	108	0.10	0.02	0.000*
	"Cerebral"	108	0.08	0.02	
	C. gaigeri/Goat	72	0.10 a	0.02	0.000**
	C. skrjabini/Sheep	36	0.10 a	0.03	
	C. cerebralis/Sheep	72	0.08 b	0.02	
	C. cerebralis/Goat	36	0.09 b	0.02	
Max. width of vas deferens of mature proglottids (mm)	"Non-cerebral"	108	0.07	0.02	0.000*
	"Cerebral"	108	0.10	0.04	
	C. gaigeri/Goat	72	0.07 b	0.02	0.000**
	C. skrjabini/Sheep	36	0.07 b	0.02	
	C. cerebralis/Sheep	72	0.10 a	0.04	
	C. cerebralis/Goat	36	0.10 a	0.04	

Table Sii (continued)

Distance between genital atrium and uterus of mature proglottids (mm)	"Non-cerebral"	108	1.23	0.12	0.007*
	"Cerebral"	108	1.29	0.18	
	C. gaigeri/Goat	72	1.22a	0.10	0.025**
	C. skrjabini/Sheep	36	1.25 a b	0.15	
	C. cerebralis/Sheep	72	1.27 b	0.17	
	C. cerebralis/Goat	36	1. 31 b	0.20	
Ratio of poral ovary lobe's area to the area through excretory canals in mature proglottids	"Non-cerebral"	108	0.05	0.01	0.000*
	"Cerebral"	108	0.04	0.01	
	C. gaigeri/Goat	72	0.05 a	0.00	0.000**
	C. skrjabini/Sheep	36	0.05 a	0.01	
	C. cerebralis/Sheep	72	0.04 b	0.01	
	C. cerebralis/Goat	36	0.04 b	0.01	
Ratio of non-poral ovary lobe's area to the area through excretory canals in mature proglottids	"Non-cerebral"	108	0.06	0.01	0.000*
	"Cerebral"	108	0.05	0.01	
	C. gaigeri/Goat	72	0.06 a	0.01	0.000**
	C. skrjabini/Sheep	36	0.06 a	0.01	
	C. cerebralis/Sheep	72	0.05 b	0.01	
	C. cerebralis/Goat	36	0.05 b	0.01	
Ratio of poral ovary lobe's area to the area of non-poral ovary in mature proglottids	"Non-cerebral"	108	0.93	0.10	0.000*
	"Cerebral"	108	0.83	0.10	
	C. gaigeri/Goat	72	0.93 a	0.09	0.000**
	C. skrjabini/Sheep	36	0.92 a	0.11	
	C. cerebralis/Sheep	72	0.83 b	0.06	
	C. cerebralis/Goat	36	0.83 b	0.10	
Ratio of vitellarium's area to the area through excretory canals in mature proglottids	"Non-cerebral"	108	0.03	0.00	0.000*
	"Cerebral"	108	0.02	0.00	
	C. gaigeri/Goat	72	0.03 a	0.00	0.000**
	C. skrjabini/Sheep	36	0.03 a	0.01	
	C. cerebralis/Sheep	72	0.02 b	0.00	
	C. cerebralis/Goat	36	0.02 b	0.00	
Ratio of cirrus pouch's length to the distance between genital atrium and uterus in mature proglottids	"Non-cerebral"	108	0.21	0.03	0.000*
	"Cerebral"	108	0.27	0.04	
	C. gaigeri/Goat	72	0.21 b	0.04	0.000**
	C. skrjabini/Sheep	36	0.22 b	0.02	
	C. cerebralis/Sheep	72	0.27 a	0.03	
	C. cerebralis/Goat	36	0.27 a	0.05	
Ratio of cirrus pouch's length to the distance between genital atrium and lateral nerve in mature proglottids	"Non-cerebral"	108	1.06	0.16	0.000*
	"Cerebral"	108	1.47	0.24	
	C. gaigeri/Goat	72	1.03 b	0.15	0.000**
	C. skrjabini/Sheep	36	1.11 b	0.18	
	C. cerebralis/Sheep	72	1.47 a	0.25	
	C. cerebralis/Goat	36	1.46 a	0.24	

Table Sii (continued)

Ratio of cirrus pouch's length to the distance between genital atrium and lateral side of the longitudinal excretory canal in mature proglottids	"Non-cerebral"	108	0.82	0.13	0.000*
	"Cerebral"	108	1.19	0.21	
	C. gaigeri/Goat	72	0.81 b	0.13	0.000**
	C. skrjabini/Sheep	36	0.86 b	0.11	
	C. cerebralis/Sheep	72	1.20 a	0.20	
	C. cerebralis/Goat	36	1.17 a	0.23	
Ratio of cirrus pouch's length to the distance between genital atrium and medial side of the longitudinal excretory canal in mature proglottids	"Non-cerebral"	108	0.67	0.10	0.000*
	"Cerebral"	108	0.88	0.16	
	C. gaigeri/Goat	72	0.65 c	0.10	0.000**
	C. skrjabini/Sheep	36	0.71 b	0.11	
	C. cerebralis/Sheep	72	0.89 a	0.11	
	C. cerebralis/Goat	36	0.85 a	0.22	
Ratio of vas deferens' length to the distance between genital atrium and uterus in mature proglottids	"Non-cerebral"	108	0.60	0.07	0.000*
	"Cerebral"	108	0.50	0.07	
	C. gaigeri/Goat	72	0.61 a	0.09	0.000**
	C. skrjabini/Sheep	36	0.60 a	0.04	
	C. cerebralis/Sheep	72	0.50 b	0.08	
	C. cerebralis/Goat	36	0.49 b	0.06	

The comparison was based on the tissue affected ("Non-cerebral" vs. "Cerebral") and parasite source (*C. gaigeri*/Goat vs. *C. skrjabini*/Sheep vs. *C. cerebralis*/Sheep vs. *C. cerebralis*/Goat).

* Significant difference between the measurements on the basis of tissue affected ($P<0.05$).

** Significant difference between the measurements on the basis of parasite source ($P<0.05$).

[1] Different letters indicate significant differences.

Table Siii. Comparison of morphological characteristics of gravid proglottids of experimentally produced *Taenia* spp.

Measurement	Tissue affected and parasite source	N	Mean[1]	Std	*P*
Area of gravid proglottids (mm^2)	"Non-cerebral"	108	21.51	4.12	0.000*
	"Cerebral"	108	14.29	8.13	
	C. gaigeri/Goat	72	21.59 a	3.94	0.000**
	C. skrjabini/Sheep	36	21.34 a	4.50	
	C. cerebralis/Sheep	72	14.29 b	8.15	
	C. cerebralis/Goat	36	14.29 b	8.21	
Area through excretory canals of gravid proglottids (mm^2)	"Non-cerebral"	108	14.84	3.48	0.000*
	"Cerebral"	108	9.93	6.16	
	C. gaigeri/Goat	72	14.87 a	3.33	0.000**
	C. skrjabini/Sheep	36	14.80 a	3.81	
	C. cerebralis/Sheep	72	9.93 b	6.18	
	C. cerebralis/Goat	36	9.93 b	6.22	
Number of branches in poral side of gravid proglottids	"Non-cerebral"	108	12.23	1.05	0.000*
	"Cerebral"	108	11.16	1.53	
	C. gaigeri/Goat	72	12.24 a	1.08	0.000**
	C. skrjabini/Sheep	36	12.22 a	0.99	
	C. cerebralis/Sheep	72	11.18 b	1.48	
	C. cerebralis/Goat	36	11.11 b	1.63	
Number of branches in non-poral side of gravid proglottids	"Non-cerebral"	108	13.23	1.63	0.000*
	"Cerebral"	108	11.46	1.56	
	C. gaigeri/Goat	72	13.24 a	1.50	0.000**
	C. skrjabini/Sheep	36	13.22 a	1.88	
	C. cerebralis/Sheep	72	11.42 b	1.54	
	C. cerebralis/Goat	36	11.56 b	1.61	

The comparison was based on the tissue affected ("Non-cerebral" vs. "Cerebral") and parasite source (*C. gaigeri*/Goat vs. *C. skrjabini*/Sheep vs. *C. cerebralis*/Sheep vs. *C. cerebralis*/Goat).

* Significant difference between the measurements on the basis of tissue affected ($P<0.05$)

** Significant difference between the measurements on the basis of parasite source ($P<0.05$)

[1] Different letters indicate significant differences.

3 Zusammenfassung

In dieser Forschungsarbeit wurden Isolate von *Taenia multiceps* als Erreger der zerebralen und nicht-zerebralen Coenurose bei Schafen und Ziegen vergleichend untersucht. Erreger von nicht-zerebraler Coenurose aus einem breiten geographischen Bereich in Afrika und Westasien wurden verglichen mit Erregern zerebraler Coenuruszysten von gesammelten Drehkrankheitsfällen aus Griechenland, wo zerebrale Coenurose häufig vorkommt, wo aber nicht-zerebrale Coenurose noch nie beschrieben wurde. Diese Forschungsarbeit enthält eine feld- und eine laborexperimentelle Komponente und beschäftigt sich mit Aspekten wie: (i) das Vorhandensein oder Fehlen von nicht-zerebraler Coenurose bei Schafen; (ii) die Beschreibung von nicht-zerebraler Coenurose im Zwischenwirt (Schafe und Ziegen); und (iii) die phylogenetische Auflösung des *T. multiceps*-Clusters sowie eine mögliche Erklärung, warum nicht-zerebrale Coenurose in bestimmten geographischen Gebieten unbekannt ist.

Im Rahmen der Felduntersuchung wurde eine Gesamtzahl von 90,415 geschlachtete Schafe und 2,284 geschlachtete Ziegen aus Schlachthöfen der Vereinigten Arabischen Emirate (UAE) und Ägypten untersucht, die aus verschiedenen tropischen und subtropischen Ländern wie Indien, Pakistan, Iran, Oman, Sudan, Somalia und Äthiopien stammten. Die Feldarbeit umfaßte außerdem die Sammlung von zerebralen Coenurosezysten von 20 Schafen und sechs Ziegen, die vom griechischen Festland und Umgebung stammten.

Im Labor wurden vier Teilstudien durchgeführt: (1) eine morphologische Untersuchung der Merkmale von nicht-zerebralen Coenurosezysten, deren Cluster und der Protoskolizes, (2) ein morphologischer Vergleich der Rostellarhaken der zerebralen und nicht-zerebralen Coenurosezysten, (3) ein morphologischer Vergleich von erwachsenen Würmern aus Hunden, die experimentell mit Protoskolizes von zerebralen und nicht-zerebralen Zysten von Schafen und Ziegen infiziert worden waren, und (4) eine molekulare Analyse von Teilsequenzen dreier mitochondrialer Gene (nad1, cox1 und 12S rRNA) der oben genannten Isolate von zerebralen und nicht-zerebralen Zysten.

Die Prävalenz von nicht-zerebraler Coenurose betrug bei Ziegen 1,75% und nur 0,008% bei Schafen. Die einzigen in den infizierten Ziegen beobachteten klinischen Auffälligkeiten waren palpierbare einzelne große Zysten in der Oberschenkelmuskulatur und eine höhere Serum-Aspartat-Aminotransferase (AST) - Aktivität. Die Zysten wurden in verschiedenen Muskeln sowie an den Nieren, am Mesenterium und am Herz gefunden; sie waren von einer faserigen, halbopaken, trübweißen Membran umgeben, die in allen Fällen einen einzelnen Coenurus enthielten. Das Ausmaß der Zysten, die Anzahl der Cluster von Protoskolizes, und die Anzahl der Protoskolizes waren bei Schafen und Ziegen ähnlich.

Sechsundsiebzig nicht-zerebrale Coenuruszysten von Ziegen wurden gesammelt und statistisch ausgewertet. Die Anzahl der Protoskolizes korrelierte signifikant positiv mit dem Volumen von Zysten (b = 6,37 > 5; R-Quadrat = 89,4%; *P* = 0,000) und die Anzahl von Clustern korrelierte signifikant positiv mit der Anzahl der Protoskolizes (b = 25,13 > 1; R-Quadrat = 79,8%; *P* = 0,000), was auf positives allometrisches Wachstum hinweist. Die Anzahl von Clustern korrelierte mit der Anzahl von Zysten (b = 0,25 < 0,5; R-Quadrat = 69,4%; *P* = 0,000), was auf ein negatives allometrisches Wachstum hinweist. Die biologische Bedeutung dieser Allometrie ist nicht bekannt, aber der Parasit investiert offenbar seine Ressourcen mehr in das Wachstum von Protoskolizes und weniger in das Wachstum von Zysten und Clustern.

Die morphologischen Untersuchungen der Rostellarhaken von Protoskolizes aus zerebralen und nicht-zerebralen Zysten und die in Hunden experimentell erzeugten adulten Würmer zeigten keine Unterschiede in Bezug auf die Wirtstierart (Schafe oder Ziegen). Im Gegensatz dazu wiesen adulte Würmer, die aus der Inokulation zerebraler und nicht-zerebraler Zysten hervorgingen, deutliche morphologische Unterschiede auf. Die meisten Meßwerte der Haken und Proglottiden unterschieden sich signifikant, aber die Form der kleinen Haken, die Verteilung der Hoden in den reifen Proglottiden und das Aussehen der Spiralen des Vas deferens waren die Merkmale mit den auffälligsten Unterschieden. Diese morphologischen Unterschiede fallen allerdings in den Bereich der Variationen von *T. multiceps.*

Die phylogenetische Analyse der mitochondrialen Haplotypen ergab drei distinkte Cluster: eines, das sowohl zerebrale Isolate aus Griechenland als auch nicht-zerebrale Isolate aus tropischen und subtropischen Ländern umfaßte, und zwei Cluster, die ausschließlich aus zerebralen Isolaten aus Griechenland bestanden. Die

meisten der nicht-zerebralen Proben gruppierten zusammen, bildeten aber keine monophyletische Gruppe. Dasselbe gilt für geographische Aspekte, obwohl Proben aus derselben Region zu Clustern tendierten. Die Daten zeigten eine hohe intraspezifische Diversität.

Die Ergebnisse dieser Studie unterstützen den Zusammenhang zwischen genetischer Identität der *T. multiceps*-Isolate und der geographischen Herkunft, und führten zum Vorschlag einer neuen Hypothese zum Vorkommen von zerebralen und nicht-zerebralen Formen. Unsere phylogenetische Analyse legt nahe, daß die Entwicklung der zerebralen Coenuri bei Schafen eine ursprüngliche Eigenschaft von *T. multiceps* sein könnte. Alle Varianten wären damit in der Lage, zerebrale Coenurose bei Schafen zu verursachen, wogegen nur einige Varianten, vor allem aus einem genetischen Cluster, sekundär die Fähigkeit erworben haben, das Gehirn von anderen Arten (Ziegen und Rindern) zu befallen, sowie nicht-zerebrale Formen (vor allem in Ziegen) hervorzubringen.

Unsere phylogenetische Analyse zeigt somit eindeutig eine molekulare Basis für nicht-zerebrale Pathogenität innerhalb der Art *T. multiceps*. Spezifische Biosicherheitsmaßnahmen sollten daher erwogen werden, um die Einführung von *T. multiceps* Varianten mit einer solchen Pathogenität zu verhindern.

Abstract

This study explores the causative agent of cerebral and non-cerebral coenurosis in sheep and goats. Cases of non-cerebral coenurosis from a wide geographical range in Africa and western Asia are investigated, and the causative agents are compared to cerebral coenurus cysts from gid cases collected from Greece, where cerebral coenurosis is common but where non-cerebral coenurosis has never been described. The study includes a field and a laboratory-experimental component and provides answers to research questions such as: (i) the presence or absence of non-cerebral coenurosis in sheep; (ii) the description of non-cerebral coenurosis in the intermediate host (sheep and goats); and (iii) the phylogenetic resolution of the *T. multiceps* cluster and a possible explanation why non-cerebral coenurosis has never been described from some geographical areas.

In the field component, a total of 90,415 slaughtered sheep and 2,284 slaughtered goats from abattoirs in the United Arab Emirates (UAE) and Egypt, originating from various tropical and subtropical countries, including India, Pakistan, Iran, Oman, Sudan, Somalia, and Ethiopia, were examined for non-cerebral coenurosis. The field component also included the collection of cerebral coenurus cysts from 20 sheep and 6 goats with cerebral coenurosis and originating from continental Greece and vicinity.

Four studies were conducted in the laboratory-experimental component: (1) a morphological study of the characteristics of non-cerebral coenurus cysts and their clusters and protoscolices, (2) a morphological comparison of the rostellar hooks of the collected cerebral and non-cerebral coenurus cysts, (3) a morphological comparison of adult worms produced in dogs experimentally infected with protoscolices from cerebral and non-cerebral cysts of sheep and goats, and (4) a molecular analysis of three partial mitochondrial genes (*nad*1, *cox*1, and 12S rRNA) of the above isolates of cerebral and non-cerebral cysts collected in Greece, UAE, and Egypt.

The prevalence of non-cerebral coenurosis was 1.75% in goats and only 0.008% in sheep. The only abnormalities observed in the infected goats were large single cysts detected by palpation in thigh muscles and higher serum aspartate aminotransferase (AST) activity. The cysts were found in various muscles and attached to the kidneys, omentum, and heart; they were surrounded by a fibrous, semi-opaque, cloudy-white membrane containing a single coenurus in all cases. The volume of the cysts, the number of the clusters of protoscolices, and the number of protoscolices were similar in sheep and goats.

Seventy-six non-cerebral coenurus cysts were collected from goats. The number of protoscolices was significantly positively correlated with the volume of cysts ($b = 6.37 > 5$; R-Sq = 89.4%; $P = 0.000$), and the number of clusters was significantly positively correlated with the number of protoscolices ($b = 25.13 > 1$; R-Sq = 79.8%; $P = 0.000$), indicating positive allometric growth. The number of clusters was significantly positively correlated with the volume of cysts ($b = 0.25 < 0.5$; R-Sq = 69.4%; $P = 0.000$), indicating however negative allometric growth. The biological significance of these allometries is not known, but the parasite may be investing its resources more in the growth of protoscolices, less in the growth of cyst volume, and even less in the number of clusters.

Our morphological studies of the rostellar hooks of protoscolices from the cerebral and non-cerebral cysts and the adult worms produced experimentally in dogs did not indicate host-adapted differences (sheep or goats). In contrast, the parasites produced by cerebral and non-cerebral cysts showed clear morphological differences. Most measurements of the hooks and proglottids differed significantly, but the shape of the small hooks, the distribution of the testes in the mature proglottids, and the appearance of the coils of the vas deferens were the most distinct characters. These morphological differences, albeit between the parasites produced by cerebral and non-cerebral cysts, fell within the range of variation of *T. multiceps.*

The phylogenetic analysis of the mitochondrial haplotypes produced three distinct clusters: one cluster including both cerebral isolates from Greece and non-cerebral isolates from tropical and subtropical countries, and two clusters including only cerebral isolates from Greece. The majority of the non-cerebral specimens clustered together but did not form a monophyletic group. No monophyletic groups were observed based on geography, although specimens from the same region tended to cluster. The recorded clusters indicated high intraspecific diversity.

The results of this study support the association between *T. multiceps* variability and the geographical origin of the isolates and lead us to propose a reformulated hypothesis for the existence of cerebral and non-cerebral forms. Our phylogenetic analysis suggests that the development of cerebral coenuri in sheep may be an ancestral property of *T. multiceps* and the main mode the parasite uses to complete its life cycle. All variants are therefore able to cause cerebral coenurosis in sheep, but only some variants, predominantly from one genetic cluster, acquired the additional capacity to affect the brain of other species (goats and cattle) or to produce non-cerebral forms, mostly in goats and more rarely in sheep.

Our phylogenetic analysis therefore clearly indicates a molecular basis for non-cerebral pathogenicity in some variants of *T. multiceps*. Specific biosecurity actions should therefore be enforced in areas where non-cerebral coenurosis does not occur to prevent the introduction of *T. multiceps* variants with such pathogenicity.

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5 Curriculum Vitae

Georgios Christodoulopoulos

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Nationality:	French and Greek
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Education

High School of Filiatra Messinie, Greece
Certificate of High School (1984), mark: 19.5/20

School of Veterinary Medicine, University of Thessaloniki, Greece
Doctor of Veterinary Medicine & Surgeon (1992), mark: 7.0/10

School of Veterinary Medicine, University of Thessaloniki, Greece
Degree of PhD in Veterinary Medicine (1998), mark: First-class Honors

Royal College of Veterinary Surgeons, Great Britain
Certificate in Sheep Health and Production (2005)

Subinstitution of Parasitology, Institute of Zoology, Faculty of Natural Sciences, University of Hohenheim
PhD candidate (2012-2017)

Languages
Greek, French, English

Computer
Computer literate

European Veterinary Colleges

European College of Bovine Health Management
Diplomate of the European College of Bovine Health Management (*de facto* recognition, 2005; recertification 2011 & 2015)

European Board of Veterinary Specialisation
European Veterinary Specialist in Bovine Health Management (2006)

European College of Small Ruminant Health Management
Diplomate of the European College of Small Ruminant Health Management (*de facto* recognition, 2011; recertification 2015)

Career

July 1992-January 1993: Clinic of Obstetrics, School of Veterinary Medicine, University of Thessaloniki (Thessaloniki, Greece)
Resident in Veterinary Obstetrics
Duties: clinical work

April 1993-August 1994: Private Veterinary Clinic (Rhodes Island, Greece)
Veterinary surgeon in general practice

September 1994-March 1998: Clinical Veterinary Medicine Department, School of Veterinary Medicine, University of Thessaloniki (Thessaloniki, Greece)
Research Assistant and Tutor in Production Animal Medicine
Duties: research/teaching

April 1998-August 1999: Private Animal Clinic "Saint Modestos" (Ekali, Attica, Greece)
Veterinary surgeon in general practice

September 1999-today: Clinical Veterinary Medicine Department, School of Veterinary Medicine, University of Thessaly (Karditsa, Greece)
Academic Staff in Production Animal Medicine (currently in the rank of Professor)
Duties: Head of Medicine/Department Chair/teaching/research/administration

Publications in the Field of Parasitology

Books

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