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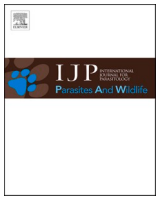

Identification of freshwater snail species and survey of their trematode infections in Ordos, China

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ABSTRACT

In order to investigate and study the species and distribution of freshwater snails in Ordos area of Inner Mongolia, as well as the trematode infection in different periods, and to provide a scientific basis for the effective prevention and control of livestock trematodiasis. In this paper, freshwater snails distributed in Ordos were widely collected for morphological identification, and PCR amplification of freshwater snails COI gene and ITS2 gene was carried out with the help of molecular biology. At the same time, microscopic examination was used to observe the trematode infection of freshwater snails in two different periods from May to July and July to September, and the molecular biology of the trematodes was identified. The results showed that the 1796 freshwater snails collected belonged to two orders, three families and four genera, i.e. *Bellamya, Radix, Galba,* and *Gyraulus*. Microscopic examination of snails showed that the infection rate of trematode larvae from July to September was significantly higher than that from May to July. The collected trematodes were identified as five species, namely *Cotylurus marcogliesei*, *Fasciola hepatica*, *Fasciola gigantica*, *Paramphistomum cervi,* and *Parastrigea robusta*. The combination of freshwater snail species in Ordos and the infection of trematode in snails showed that a large number of freshwater snails were infected with trematodes, especially from July to September, when there is more rain and suitable climate, which causes serious harm to local livestock.

1. Introduction

Freshwater snails, a major category of medical shellfish studies (Hu et al., 2022), are gastropods that live in freshwater and have an important economic and pharmacological value. Freshwater snails also play an important role as intermediate hosts in the life cycle of many parasites and may pose a risk to the health of animals and human beings (Barton et al., 2022; Lu et al., 2018). Zoonoses among the diseases transmitted by freshwater snails have been recognised as an important component of several parasitical diseases (Chontananarth et al., 2017). Some species of freshwater snails being an important intermediate hosts of trematode parasites (Borgsteede, 2004; Shamsi et al., 2023).

Trematodes are a highly diverse phylum of parasitic flattened animals, with about 18,000 species existing (Hammoud et al., 2022), which are parasitic diseases of humans and livestock. Lu et al. (2018) reported that millions of people in about 90 countries suffer from parasitic diseases transmitted by freshwater snails, which are vectors and intermediate hosts. The main trematodes parasitic in freshwater snails and capable of infecting humans are *F. hepatica*, *P. westermani*, *F. buski*, *S. mansoni,* and *S. japonicum*. Globally, more than 20 million people are infected with *F. hepatica* (Correa et al., 2011) and *P. westermani* (Hong et al., 2019), and more than 200 million people are infected with *Schistosoma* (Shan et al., 2020; Skelly and Da'Dara, 2023; Zheng, 2021). Trematode infections pose a public health risk and cause serious socio-economic problems in many tropical and subtropical countries (Prasopdee et al., 2015), including Viet Nam, Myanmar, Cambodia, Laos, and Thailand. China met the transmission control standard for *schistosomiasis* in 2015 and is now moving towards the elimination stage (Dai et al., 2019), but as one of China's "five major parasitic diseases" (Li, 1996), trematode parasitosis is still a serious foodborne parasitic disease in China.

Ordos is located in the southwestern part of Inner Mongolia Autonomous Region, surrounded by the Yellow River on the north, west and east sides, with more wetlands and beachlands in some areas. These areas are perennially waterlogged with lush pasture, providing good living conditions for the survival of freshwater snails. The distribution of

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freshwater snails is correlated with parasitic diseases to a certain extent. Investigating and understanding the species of freshwater snails and the infection of trematodes in different periods in the Ordos region can help to better detect and predict the prevalence of these trematodes, as well as to prevent some trematodiasis in humans and livestock (Nguyen et al., 2021; Prastowo et al., 2022; Wiroonpan et al., 2021).

2. Materials and methods

2.1. Collection of sample

Collection of freshwater snails: The collection was completed from July 2022 to July 2023. According to the climatic characteristics of Ordos, we divided the collection time into two periods: July to September 2022 and May to July 2023. A total of 1796 freshwater snails were collected from the shoals, lakes, low-lying and stagnant puddles depressions of various banner counties in the Ordos region.

Collection of trematode larvae in freshwater snails: Clean the snails and put them between two slides and squeeze them, pick off the shells and observe under the microscope. Rinse the positive snail with water, filter out the snail meat and impurities with a sieve, suck the cercariae, metacercariae and sporocyst into the centrifuge tube with a pipette, centrifugation for a short period of time, and then discard the upper layer of aqueous solution and put precipitate into liquid nitrogen to store for spare.

2.2. Morphological identification of the freshwater snails

The collected snails were fully cleaned of the soil on their shells, and the initial classification of snails was made by observing the colour and pattern of their shells with the naked eye. The shell peristoma height, shell width and shell peristoma width of each type of snails were measured with vernier calipers, and the collected freshwater snails were identified morphologically through the measured data and morphological characteristics, in combination with the identification methods of Liu (1979) and Li (1956).

2.3. Trematode larvae infections in freshwater snails

Two slides were used to squeeze the snail shells and flatten them, then the crushed shells were peeled off from the snail flesh with forceps, and the snail flesh was observed under the microscope for the presence of trematodes. With a picking needle, the trematode was picked out of the conch into a dish containing saline flat, and then placed on a clean slide, the morphology was observed under a microscope and photographed for retention.

SPSS 25.0 software was used to analyze the chi-square test of the positive rate of freshwater snail trematodes in two different time periods in Ordos area, and the difference was statistically significant with $P < 0.05$.

2.4. Molecular identification of freshwater snails and trematode larvae in freshwater snails

2.4.1. Primer design and synthesis

Freshwater snail species identification Based on the published COI gene sequences and ITS2 gene sequences of freshwater shellfish in Genbank, the application software was used to design specific primers. COI (Folmer et al., 1994) gene: CO1–F: 5′-GGTCAACAAATCATAAAGATATTGG-3′, CO2-R: 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′. ITS2 (Kane and Rollinson, 1994) gene: ETTS1-F: 5′-TGCTTAAGTTCAGCGGGT-3′, ETTS10-R: 5′-GCATACTGCTTTGAACATCG-3′, the primers were synthesised by BGI Biologics. Molecular identification of trematodes in freshwater snails was performed by selecting trematode universal primer ITS2 gene sequences (Sugiyama et al., 2002). 3 S–F: 5′-GGTACCGGTGGATCACTCGGGCTCGTG-3′, A28-R: 5′-GGGGATCCTGGTTAGTTTTTCTTTTCCTCCGC-3′, and the primers were synthesised by BGI Biologics.

2.4.2. DNA extraction

DNA extraction from freshwater snails: Snail tissue samples were taken and the DNA of snails was extracted according to the Marine Animal Tissue Genomic DNA Extraction Kit (Centrifugal Column Type) produced by TIANGEN, and the concentration of DNA was determined and stored in the refrigerator at −20 °C.

DNA extraction from trematode larvae: Trematode samples were taken and trematode DNA was extracted according to the Tissue DNA Extraction Kit produced by ABclonal, the DNA concentration was determined and stored in the refrigerator at −20 °C.

2.4.3. PCR amplification and sequencing

PCR amplification and sequencing of freshwater snails: the amplification of COI and ITS2 genes was carried out according to the designed specific primers. The PCR amplification system was as follows: 9 μL of ddH_2O, 8 μL of PCR Mixture, 2 μL of DNA template, and 0.5 μL of each of the upstream and downstream primers. The conditions of PCR amplification of COI genes were as follows: pre-denaturation at 95 °C for 5 min, denaturation at 94 °C for 15 s, annealing at 45.2 °C for 55 s, extension at 72 °C for 1 min, and extension at 72 °C for 7 min. The PCR amplification conditions of ITS2 gene were as follows: pre-denaturation at 95 °C for 5 min; denaturation at 94 °C for 30 s; annealing at 46 °C for 45 s; extension at 72 °C for 55 s; 35 cycles; extension at 72 °C for 7 min. The PCR amplification products were detected by 1 % agarose gel electrophoresis, and the size of the target fragment of the COI gene product was 710 bp, which corresponded to the size of the ITS2 gene sequence of the sample was about 500 bp. The verified correct PCR products were gel recovered and sent to Beijing BGI Biologics for first-generation sequencing.

PCR amplification and sequencing of trematode larvae: The ITS2 gene was amplified according to the specific primers designed. The PCR amplification system was as follows: 9.5 μL of dd H_2O, 12.5 μL of PCR Mixture, 2 μL of DNA template, and 0.5 μL of each of the upstream and downstream primers. The conditions of the PCR amplification of the ITS2 gene were as follows: pre-denaturation at 94 °C for 5 min; denaturation at 94 °C for 45 s; annealing at 95 °C for 45 s; extension at 72 °C for 1 min in 32 cycles; and extension at 72 °C for 10 min. The PCR amplification products were detected by 1.5% agarose gel electrophoresis, and the size of the ITS2 gene sequence of the corresponding samples was around 600 bp. The verified correct PCR products were gel recovered and sent to Beijing BGI Biologics for bidirectional sequencing.

3. Results

3.1. Morphological identification and molecular biology identification results of freshwater snails

After morphological and molecular biological identification, the 1796 freshwater snails collected were identified into two orders, three families and four genera, namely *Mesogastropoda, Basommatophora*, *Viviparidae, Lymnaeidae, Planorbidae*; *Bellamya, Radix, Galba, Gyraulus* (Fig. 1). Among them, the genus *Galba* is the dominant species in the Ordos region, accounting for a relatively large proportion.

Bellamya aeruginosa (Fig. 1a): *Mesogastropoda Viviparidae Bellamya,* shell height about 25 mm, width about 15 mm, long conical shape, with 6–7 spiral layers; The spiral part is pointed conical rows, and the body spiral layer is slightly enlarged; The shell surface is smooth and patina or greenish-brown. *Radix plicatula* (Fig. 1b, c): *Basommatophora Lymnaeidae Radix,* the shell is large, the chitin is thin, and the body spiral layer is enlarged. *Gyraulus convexiusculus* (Fig. 1d): *Basommatophora Planorbidae Gyraulus,* the shell is small, the diameter is generally about 8 mm, the height of the shell is 1.5 mm, the chitin is thin, there are 4–5 spiral layers, the same spiral layer can be seen above and below the shell, the

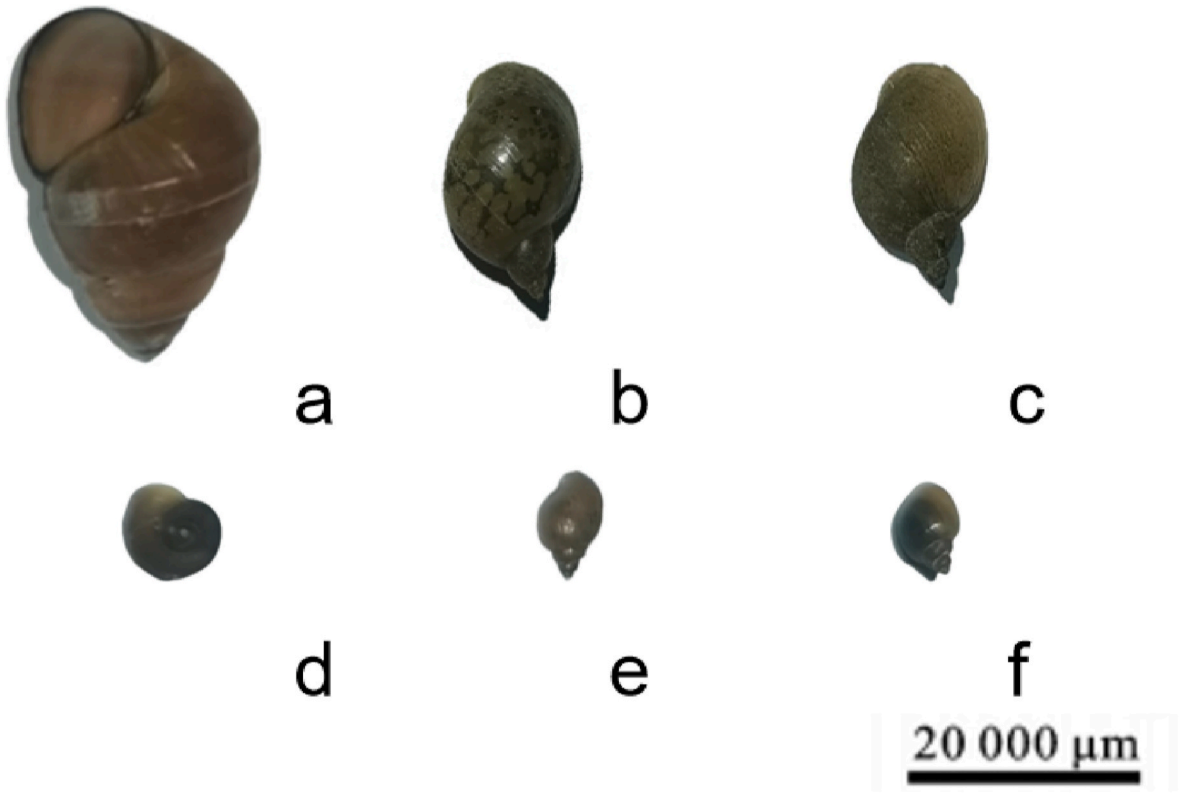

Fig. 1. Morphological characteristics of major freshwater snails in the Ordos area. a: *Bellamya aeruginosa*; b, c: *Radix plicatula*; d: *Gyraulus convexiusculus*; e, f: *Galba pervia*.

center of both sides is concave, the shell surface is gray, gray-yellow or light brown, often covered with black shell skin. *Galba pervia* (Fig. 1e, f): *Basommatophora Lymnaeidae Galba,* the shells are small thin shell and inflated oval, shell shape with 4–5 spiral layers.

3.2. Infection of freshwater snail trematode

In this study, 905 freshwater snails were collected from July to September 2022 and 891 freshwater snails from May to July 2023. There was no change in the species of freshwater snails collected in the two periods, and the proportions of various types of freshwater snails collected in the two periods were close to the same. The positive rate of trematode infection was observed by microscopic examination. Trematodes are found in the snail body, including cercariaes, metacercariae, and sporocyst. From July to September, freshwater snails had the highest rate of trematode infection, which was 62.1%. The infection rate of trematodes from May to July was lower than that from July to September, with an infection rate of 30.9%. According to Chi-square test, there were statistically significant differences in the positive rate of freshwater snails in different time periods in Ordos ($\chi^2 = 176.027$, $p < 0.001$) (Table 1).

Table 1
The positive rate of freshwater snails in different time periods in Ordos area.

		Infected or not		Total	positive rate
		Infected	Not infected		
Period	7–9	562	343	905	62.1%
	5–7	275	616	891	30.9%
Total		837	959	1796	46.6%

3.3. Molecular biological identification results of trematodes larvae

A total of five ITS2 gene sequences of the expected size (around 600 bp) were amplified from seven samples, and five species of trematodes were identified, namely *Cotylurus marcogliesei*, *Fasciola hepatica*, *Fasciola gigantica*, *Paramphistomum cervi*, *Parastrigea robusta* (Fig. 2) (Table 2)

Cercariae (Fig. 2A–D) were collected from *Galba pervia*, and showed obvious small spines, and the length of the body is similar to the tail. Cercariae (Fig. 2E–G) were collected from *Radix plicatula* have bifurcated tails that are significantly longer than the body, and the body length is about 1/3 of the tail. Sporocyst (Fig. 2H, I) are cylindrical at both ends, the body is curved to the ventral surface, moving slowly, and only slightly peristalsis. The metacercariae (Fig. 2J, K) collected from *Galba pervia* and *Radix plicatula* are two forms: round and calabash.

4. Discuss

Correct identification of freshwater snails as well as trematode larvae in snails is essential to determine the epidemiological risk area of trematodes (Ferreira et al., 2021). Currently, most of the freshwater snail species investigations are based on morphological identification. Li et al. (1998), Guo et al. (2009) and Wang et al. (2010) conducted morphological identification of freshwater snails in Gannan pastoral areas, Beijing and the Tarim region successively. However, some snails have high genetic variation, and it may be difficult to accurately differentiate their species relationships using traditional morphological identification (Alharbi et al., 2022). Meanwhile, the morphology of each trematode larva is similar, and it is difficult to differentiate trematode species. So it is necessary to accurately identify freshwater snails and their internal trematode larvae with the help of molecular biology methods for species identification (Chontananarth and Wongsawad, 2010). Therefore, in this study, we used a combination of morphological identification and molecular biology identification to identify the

Table 2
Identification of trematodes larvae observed in freshwater snails.

Species Name	Worm Stage	Host	Collection Place	Genbank
Paramphistomum cervi	metacercariae	*Gyraulus convexiusculus, Galba pervia*	Wushen Banner, Ejin Horo Banner, Dalad Banner	KJ459936.1
Fasciola hepatica	Cercaria; sporocyst; metacercariae	*Galba pervia, Radix*	Wushen Banner, Ejin Horo Banner, Dalad Banner	MN97007.1
Fasciola gigantica	Cercaria; sporocyst; metacercariae	*Galba pervia, Radix*	Wushen Banner; Otog Front Banner, Hangjin Banner,	MT429176.1
Parastrigea robusta	Cercaria	*Radix*	Wushen Banner	MF537205.1
Cotylurus marcogliesei	Cercaria; sporocyst; metacercariae	*Radix*	Wushen Banner	MH521248.1

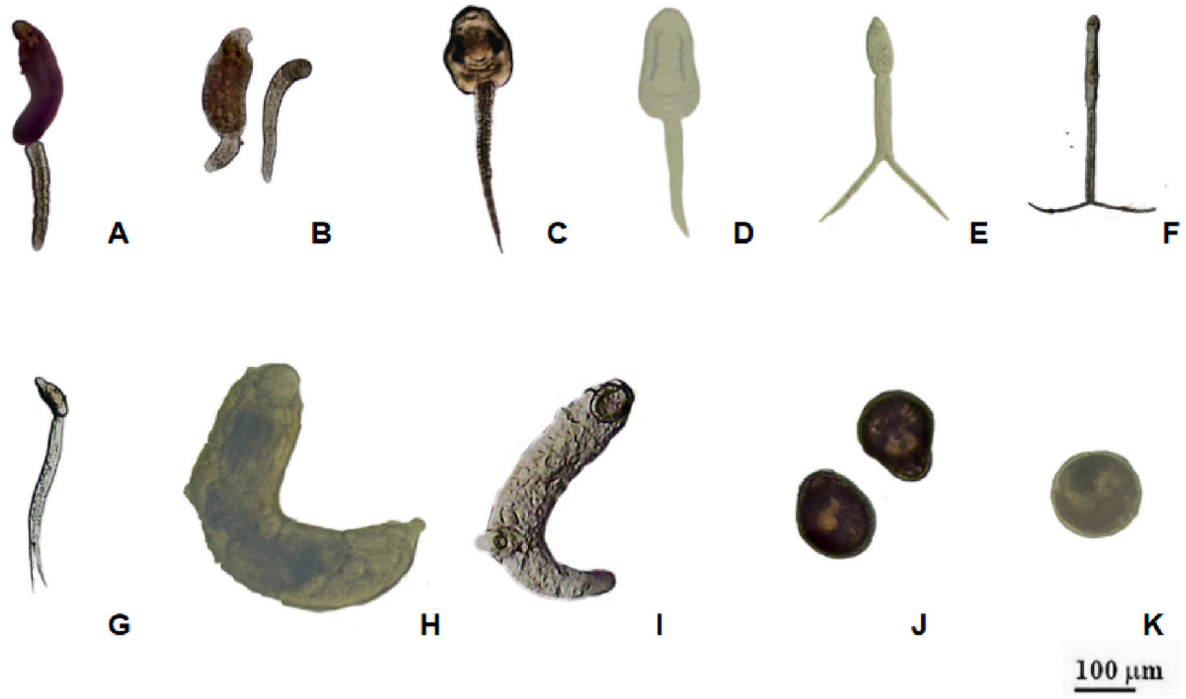

Fig. 2. Trematodes larvae in freshwater snails A–G: Cercaria; H, I: sporocyst; J, K: metacercariae.

species of freshwater snails in Ordos region, and molecular biology methods to identify the species of trematode larvae in Ordos.

Compared with the results of Collado et al. (2020), Zikmundova et al. (2014) and Dung et al. (2013), the 1796 freshwater snails collected in this study were identified into two orders, three families and four genera, which confirmed the abundance of freshwater snails in the Ordos region of Inner Mongolia. These freshwater snails are important intermediate hosts for some trematodes. In 2017, Chontananarth et al. (2017)detected 12 species of trematode larvae in freshwater snails in Nakhon Nayok Province, Thailand. Compared with the results of this study, a total of five trematode larvae were found in freshwater snails in Ordos area, namely *Fasciola hepatica, Fasciola gigantica*, *Paramphistomum cervi*, *Cotylurus marcogliesei,* and *Parastrigea robusta*. In 2022, Bawm et al. (2022) used freshwater snails in different regions as a whole to detect the infection rate of trematodes, and this study detected the infection rate of trematodes based on freshwater snails in several banner counties in Ordos region at different periods. As compared to the findings of Barton et al. (2022), the *Galba pervia* were predominant among the freshwater snails collected in the present study and the *Galba pervia* had the highest prevalence of infection as compared to other snails. The results of Roessler et al. (2022) showed that the *Galba pervia* is the main intermediate host of *Fasciola hepatica*, which is in agreement with our findings, and some cattle and sheep were also found to infected *fascioliasis* in Ordos region. Therefore, the risk of *fascioliasis* in human and livestock is high in Ordos region. In addition, the cercariae of *Fasciola gigantica* and *Paramphistomum cervi* were isolated from the snails. These two common parasites can infect cattle and sheep (Hambal et al., 2020), affect the health of cattle and sheep, and in severe cases, can lead to the death of the diseased cattle and sheep and pose a great danger to the local animal husbandry industry. The other two trematodes, *Cotylurus marcogliesei* (Suleman, 2019) and *Parastrigea robusta* (Heneberg et al., 2018), primarily infect birds, don't pose a risk to the local animal husbandry industry and have not been previously reported.

The Ordos region has a typical temperate continental climate. In the spring, the temperature began to rise, and in late spring, we found that freshwater snails have appeared in the water areas such as shoals and lakes. The summer is warm, the rain is relatively concentrated, and the annual precipitation is concentrated in July to September, during which freshwater snails begin to multiply in large numbers. Autumn and winter are not suitable for freshwater snail collecting due to the rapid drop in Autumn temperature, early frost, and long and cold winter. Therefore, according to the climatic characteristics of Ordos, we chose the two time periods of May–July and July–September to collect freshwater snails intensively. We conducted statistics on the positive rate of freshwater snails in 7 banner counties and two time periods in Ordos region. It was found that the trematode positive rate of freshwater snails in July to September was significantly higher than that in May to July, which was likely related to local temperature and rainfall. The temperature in Ordos is about 15–27 °C from May to July, and about 25–36 °C from July to September. The lower temperature from May to July compared to July to September is likely to affect the reproduction of some trematodes, and it has been confirmed that temperature affects the growth and reproduction of trematodes (Larsen and Mouritsen, 2014; Poulin, 2006; Resetarits and Byers, 2023). In addition, the rainfall from July to September is heavy, and the grassland is waterlogged to form a lot of shoal, which is conducive to the reproduction of freshwater snails,

resulting in a high positive rate of snail trematodes.

In addition, in freshwater snails, we found that some of the cercariae tail had already broken off inside the snail and formed fresh, milky-white "gourd-type" metacercariaes. This is different from the cercariae that overflowed out of the snail and developed into metacercari in the water as reported by Groning et al. (2023).

This study suggests that freshwater snails in Ordos region are mainly intermediate hosts of *Fasciola hepatica, Fasciola gigantica* and *Paramphistomum cervi.* These trematodes mainly harm the development of local cattle and sheep industry, and freshwater snails play an important role in the life history of these trematodes. Therefore, elimination of the intermediate hosts is one of the most important means of preventing trematode infection in local cattle and sheep (Bawm et al., 2022; King et al., 2015; Tookhy et al., 2023). Currently, chemical snail elimination, i.e., drug snail elimination, is mostly used internationally, e.g., clonidine is the only commercially available snail elimination drug recommended by the World Health Organisation (Konan et al., 2022). It is understood that local herders in Ordos have relatively little knowledge of snail infection parasites and don't have the habit of snail extermination. Most of them use 1% copper sulphate under the guidance of local Animal Disease Prevention and Control Centre to spray the snail on the pasture wetlands and shoals. Therefore, it is necessary to formulate and carry out the precise prevention and control of bovine and sheep trematode disease from the aspects of scientific snail extermination and reasonable medication.

5. Conclusions

The species of freshwater snails in Ordos area of Inner Mongolia are abundant, mainly *Bellamya aeruginosa, Radix plicatula, Gyraulus convexiusculus,* and *Galba pervia.* The rate of trematode infection with freshwater snails in July to September was higher than that in May to July, and it was statistically significant. The main types of snail trematode infection include *Cotylurus marcogliesei, Fasciola hepatica, Fasciola gigantica, Paramphistomum cervi,* and *Parastrigea robusta*, indicating that freshwater snails in the Ordos region can be used as an intermediate host for a variety of trematodes and epidemic with a variety of trematodias.

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Author contributions

Conceptualization, S.H.; methodology, N.L.; validation, S.H., and N. L.; formal analysis, N.L.; investigation, Y.H. and B.B; resources, Y.H., B. B., B.H., W.T. and S.L.; data curation, Y.H.; writing—original draft preparation, N.L.; writing—review and editing, S.H.; visualization, N.L.; supervision, S.H.; project administration, S.H.; funding acquisition, S.H. All authors have read and agreed to the published version of the manuscript.

Data availability statement

No additional data are associated with this study.

Declaration of competing interest

The authors have no conflicts of interest.

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