First report of Thelazia callipaeda infection in Phortica okadai and wildlife in national nature reserves, China

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Short report

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Abstract

**Background:** *Thelazia callipaeda* is a zoonotic parasitic nematode of the family Thelaziidae, with *Phortica okadai* as the intermediate host and the only confirmed vector in China. China has the largest number of cases of thelaziosis in humans of the world. It is generally believed that domestic animals (dogs and cats) are the most important reservoir hosts of *Thelazia callipaeda* and directly threaten humans. At present, there is not much research and attention on the role of wildlife in the transmission cycle of thelaziosis in wildlife nature reserves.

**Methods:** During 2016-2019, we selected four wildlife national nature reserve across the country as monitoring points for *Phortica okadai* and wildlife, and we chose to use fly-trap method for monitoring *Phortica okadai* density. Morphological analysis of the parasites collected from the conjunctival sac of the wildlife was taken as the first step, and a specific PCR was used for exact confirmation.

**Results:** In 2019, the density of *Phortica okadai* in Foping National Nature Reserve of China, increased sharply and infected *Phortica okadai* were newly found in wildlife nature reserves. Wild giant panda, wild boar, leopard cat, and black bear were newly found to be infected by *Thelazia callipaeda* (one animal of each species). A total of four worms were collected and one worm was collected from each animals. The morphologic characteristics of the four worms led to their identification as *Thelazia callipaeda*, which was molecularly confirmed by a specific PCR amplification.

**Conclusions:** This is the first report of *Phortica okadai* as well as a variety of wildlife including wild giant panda infected by *Thelazia callipaeda* in wildlife nature reserves in China. This indicates that there has been a transmission cycle of thelaziosis among wildlife in wildlife nature reserves. The increasing number of case reports in wildlife suggests the likely risk of infection of *Thelazia callipaeda* in villagers around wildlife nature reserves.

**Background**

*Thelazia callipaeda* is a zoonotic parasitic nematode of the family Thelaziidae, with *Phortica okadai* (Diptera, Drosophilidae, Steganinae) as the intermediate host and the only confirmed vector in China [1-5]. The definitive host span is large and can be infected in wildlife, domestic animals and humans [6]. *T. callipaeda* is also a kind of parasitic nematodes that parasitize the conjunctival sac and lacrimal duct of mammals. Its reproductive mode is ovoviparous [7]. It takes *Phortica sp* as their intermediate host and mammals as their definitive host, establishing a transmission cycle between mammals and flies. Dogs are the most important reservoir hosts. When *P. okadai* lick the mammal's eye, the infective larvae of *T. callipaeda* escape from the *P. okadai* 's proboscis and invade the conjunctival sac of the mammal [4]. The damage caused by parasitic *T. callipaeda* on the eyes of animals is different in severity. The damage mechanism is the friction of sharp ring folds on the surface of the worm and the mechanical damage of the eye tissue caused by the adsorption of the mouth sac. In addition, adult worm secretions and excreta stimulate the tissues of the eyes. In clinically, some infected dogs show foreign body sensation, which increases secretions, eyelid edema, conjunctival congestion, inflammation or formation of small ulcers, turbidity of aqueous compartment, and increases intraocular pressure [7,8].

In recent years, many countries have reported cases of human infection with *T. callipaeda*, and in Italy, Germany, Serbia and other European countries, there are also reports of dogs and wild hosts infected with *T. callipaeda* [9-11]. From 1917 to 2019, 643 cases of thelaziosis in humans have been recorded in China [12,13]. China has the largest number of human cases of thelaziosis in the world. It can be seen that the number of infection cases has increased significantly since 1970, and although the number of cases has decreased compared with that from 1970 to 1999 in the past 20 years, it still tends to increase again [12,13]. The increase in the number of *P. okadai*, coupled with the proliferation of domestic animals (dogs and cats), has led to increased vigilance against *T. callipaeda* infections.

The infection rate of villagers is obviously higher than that of urban people [13]. This is because the villages environment is more suitable for the survival of *P. okadai*. From 2016 to 2019, we performed ocular examination of domestic animals (dogs) in the villages around wildlife nature reserves and found that the prevalence rate, in 2019, was as high as 84.62% (88 of 104), higher than that in 2016 (38.05%, 43 of 113), 2017 (53.92%, 55 of 102) and 2018 (56.25%, 63 of 112).
However, most of wildlife nature reserves are surrounded by villages. Moreover, the number of wildlife in the wildlife nature reserves is far more than the number of domestic animals in the surrounding villages. If the wildlife spread the *T. callipaeda* on a large scale, it will be a threat to the villagers and domestic animals.

In order to understand the prevalence of *T. callipaeda* in the wildlife nature reserves and surrounding villages, we selected four wildlife national nature reserve across the country as monitoring points for *P. okadai* and wildlife, during 2016-2019. The four monitoring points are located in the wild giant panda home range, one of the most complex topographical regions in the world with the most obvious vertical zoning of climate, and home to more than 8000 confirmed species of wildlife and plants. There are villages around these reserves, and some villagers raise domestic animals. These areas have overlaps between the activities of wildlife and domestic animals (dogs and cats) [14]. In addition, these reserves were also selected because they are close to densely populated cities.

**Methods**

**Monitoring points for *P. okadai* and wildlife**

During 2016-2019, we selected four wildlife national nature reserves across the country as monitoring points for *P. okadai* and wildlife. They are Foping National Nature Reserve, Shaanxi, China (FNNR, 33°38′43″N, 107°47′38″E), Tangjiahe National Nature Reserve, Sichuan, China (TNNR, 32°34′44″N, 104°45′43″E), Wolong National Nature Reserve, Sichuan, China (WNNR, 31°02′20″N, 103°11′52″E) and Fengtongzhai National Nature Reserve, Sichuan, China (FTZNNR, 30°22′05″N, 102°48′52″E) (Fig. 1).

**Monitoring *P. okadai* density and morphological identification**

According to the "Surveillance methods for vector density – Fly" promulgated by the National Health Commission of the People's Republic of China [15], we chose to use fly-trap method for monitoring *P. okadai* density. 200 traps were placed at each monitoring point, of which 100 were distributed in wildlife home range and the other 100 were distributed in surrounding villages. Prepared fruit mash was put in the traps. Monitoring was done from April to October. The fly traps were placed at 9 am in the middle of each month, and were taken back at 9 am the next day. Because there are many kinds of flies in the traps, *P. okadai* can be identified according to the morphological characteristics. They were taken out for further morphological analysis under microscope. The density of *P. okadai* was calculated and the infection was identified by dissection. Density represents the number of *P. okadai* per cage for 24h [15].

**Sample collection**

In 2019, four cases of ocular worm infection in wild giant panda (Additional file 1: Figure S1), wild boar, leopard cat and black bear (one animal of each species) were found during ophthalmic examination under injection anesthesia in FNNR. A total of four worms were collected and one worm was collected from each animal.

**Parasite collection and treatment**

The worms were removed from the conjunctival sac and placed in a sampling tube containing 70% ethanol. Morphological analysis of the parasites collected was taken as the first step, and a specific PCR was used for exact confirmation.

**Morphological analysis**

The parasites collected from the eyes of each animal were identified on the basis of morphologic keys under a light microscopy combined with a camera.

**Sequence and Phylogenetic analysis**

We extracted genomic DNA of each worm from the conjunctival sac of wild giant panda, wild boar, leopard cat, and black bear with the HiPure Tissue & Blood DNA Kit (MAGEN, China). A partial sequence of the mitochondrial cytochrome c oxidase subunit 1 gene (cox1, 689 bp) was amplified by PCR. Amplicons were purified by using HiPure Gel Pure Micro Kit (MAGEN, China) and sequenced in an ABI3730XL by using the BigDyeTr v3.1 Cycle Seq Kit (Applied Biosystems, USA). Amplicon sequences were
determined in both directions (GenBank accession nos. MN719908, MN719912, MN719913 and MN719914) and performed genetic analyses using available sequences of related nematodes from GenBank and the GISAID database (https://www.gisaid.org). The phylogenetic tree (Fig. 2) was constructed by using MEGA version 6 (https://www.megasoftware.net) by the neighbor-joining method with 1,000 bootstrap replicates [16-19].

Results

Morphological examination under microscope (Additional file 1: Figure S2) showed characteristic features of *P. okadai* [2,5,20], however the genitalia were not examined.

It can be seen from our monitoring data (Table 1- Table 4) that before 2019 (in 2016, 2017 and 2018), there were fewer *P. okadai* in wildlife home range than in surrounding villages, and no infected *P. okadai* were found in the wildlife home range. Only in surrounding villages, a large number of infected *P. okadai* were found. However, in 2019, the density of *P. okadai* in FNNR increased sharply and infected *P. okadai* were newly found in wildlife home range of FNNR. July and August were the two months with the highest distribution density of *P. okadai*, and also the two months with the highest number of *P. okadai* with larvae of *T. callipaeda* (Fig. 3a).

Through morphological observation of the collected parasites, it could be seen that the four worms have cylindrical shape with thin ends, milky white and slightly transparent. All of worms were females. The mean body length and width of the female were 13.7mm and 0.36mm respectively. Under the optical microscope, the buccal capsule, pharynx, and esophagus of the anterior segment of the worm body and its serrated, wrinkled surface and the caudal end of the worm were visible (Fig. 3b, Fig. 3c). In the midsection of the female's uterus, there were larvae and oval eggs (Fig. 3d). According to the morphological characteristics of the worm, it could be identified as *T. callipaeda*.

By the 2% agarose gel electrophoresis, the PCR amplified products of each sample showed a DNA band of about 689 bp length, which was consistent with the expected size (Fig. 4). There were no nonspecific bands. According to alignment and phylogenetic analysis, the neighbor-joining method confirmed that the *T. callipaeda* cox1 sequences in wild giant panda, wild boar and leopard cat (GenBank accession nos. MN719908, MN719912 and MN719913) obtained clustered with those of *T. callipaeda* in dogs, cats, and humans from China, Japan and Korea. The sequences from black bears (GenBank accession nos. MN719914) were closely related to those from European animals, as they were clustered together. All cox1 sequences of *T. callipaeda* were clustered into one large branch, while the cox1 sequences of *Onchocerca lupi* from USA represent an outgroup (Fig. 2).

Through morphological observation and cox1 gene detection, we can confirm that all four ocular worms (one worm was collected from each animals) infected with wild giant panda (Additional file 1: Figure S1), wild boar, leopard cat and black bear (one animal of each species) are *T. callipaeda*.

Discussion

It is generally believed that domestic animals are the most important reservoir hosts of *T. callipaeda* and directly threaten human [21-26]. However, in 2019, wild giant panda, wild boar, leopard cat, and black bear have been found to suffer from the thelaziosis. In 2019, through the investigation of the intermediate host, it was found that there were a large number of infected intermediate hosts in wildlife home range of FNNR. We believe that thelaziosis has already spread into the wildlife nature reserves. The outbreak of infection of *P. okadai* in wildlife home range of FNNR shows that this transmission cycle has been established.

Summer is the most active season for the intermediate host of *T. callipaeda* [27], it is also the season for numerous wildlife species to migrate in the wildlife nature reserves [14,28-30]. The newborn larvae of *T. callipaeda* are directly produced in the conjunctival sac of wildlife. When the intermediate host, *P. okadai*, licks the wildlife's eye secretions, it sucks the newborn larvae into its body. After ecdysis of the larvae 2 times, the newborn larvae develop into infective larvae and then enter the head and proboscis of the *P. okadai*. When *P. okadai* lick the another specimen of host, the infective larvae of *T. callipaeda* escape from the *P. okadai*’s proboscis and invade the conjunctival sac of the wildlife, it usually takes 15 to 20 days to develop into an adult (7). The female *T. callipaeda* can continue to produce larvae till it is about 35 days old, which are then licked by the intermediate host, *P. okadai*, and the cycle continues. The infected *P. okadai* threaten domestic animals and villagers.
Once thelaziosis is widely spread among wildlife, with the frequent villager activities around the wildlife home range, it will inevitably threaten the human beings in turn. If the infection is not controlled, the infected wildlife gradually forms a reservoir host bank among wildlife. When the winter is approaching, wildlife migrates to the villages, causing interactions between wildlife and domestic animals [14,28-30]. The interaction creates an opportunity for the transmission of potential *T. callipaeda* between wildlife and domestic animals by *P. okadai*.

*P. okadai* of FNNR has the highest density as compared to other studied wildlife biotopes, which is related to its geographical environment and climate [27]. It is located in Foping county, in the northeast of Hanzhong city, Shaanxi province, belonging to the north sub-tropical climate zone, Qinling mountains in the north and Daba mountains in the south are two barriers, with an altitude of 980-2904 meters. The humid air does not spread easily to the north. The climate is mild and humid. The suitable climate provides a good habitat for *P. okadai*, which is the vector of *T. callipaeda*. No *P. okadai* is found in the WNRR monitoring points. The reason is that the altitude of most local mountain peaks is over 4000 meters and the average annual temperature is 8.5±0.5°C, which seems too low for the survival of *P. okadai*.

In this study, only four *T. callipaeda* were collected from the eyes of four wildlife, and one worm was collected from each animals. Due to the wide living range of wildlife and irregular activities, the wildlife must be anesthetized for each collection, which brings great difficulties to the collection work. Therefore, in order to understand the actual number and species of wildlife infected, we need to continue to collect and screen in the future.

Although no wildlife infection cases have been presently found in the three reserves other than FNNR, the monitoring of the intermediate host needs to be carried out all the time. At the same time, the domestic animals in villages should be given deworming medicine regularly. Such efforts could monitor the epidemic trend of thelaziosis and weaken the threat to the public.

**Conclusions**

To the best of our knowledge, this is the first report of *P. okadai* as well as a variety of wildlife including wild giant panda infected by *Thelazia callipaeda* in wildlife nature reserves in China. This study illustrates the importance of wildlife in vector-borne zoonosis. Further studies need to focus on the assessment of the risk of *T. callipaeda* infection in villagers around the wildlife nature reserves.

**Declarations**

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**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets supporting the conclusions of this article are included within the article. Sequences obtained during the current study are available in the GenBank database with accession numbers MN719908, MN719912, MN719913 and MN719914.

**Competing interests**
The authors declare that they have no competing interests.

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Not applicable.

**Authors' contributions**

YPJ, ZCL, DGL, and JHL conceived the study. YPJ and ZCL organized the sampling plan. ZCL, JQW, YFW, NJH and LBT were responsible for the collection of cases of wildlife thelaziosis used in this study. ZCL obtained the sequence of *Thelazia callipaeda* and analysed the results. All of members participated in the capture of *Phortica okadai* in wildlife nature reserve. YPJ and ZCL drafted the manuscript and all authors critically contributed to its final version. All authors read and approved the final manuscript.

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Tables
Table 1 The density and infection situation of *Phortica okadai* in wildlife home range and surrounding villages, China, 2016

| Monitoring points | Region       | Months | No. Fly Traps | No. *P. okadai* | Fly density (fly/cage /24h) | No. infected *P. okadai* |
|-------------------|--------------|--------|---------------|----------------|-----------------------------|--------------------------|
|                   |              |        | No. Fly Traps | No. Fly Traps |                            |                          |
|                   | FNNR         | April  | 100           | 100            | 141                         | 85                       | 1.41                      | 0.85                      | 69                       | 0                        |
|                   |              | May    | 203           | 203            | 2.03                        | 1.01                      | 111                       | 0                         |
|                   |              | June   | 202           | 202            | 2.02                        | 1.01                      | 111                       | 0                         |
|                   |              | July   | 206           | 206            | 2.06                        | 1.23                      | 112                       | 0                         |
|                   |              | August | 221           | 221            | 2.21                        | 1.24                      | 126                       | 0                         |
|                   |              | September | 218      | 218            | 2.18                        | 1.19                      | 102                       | 0                         |
|                   |              | October | 137          | 137            | 1.37                        | 1.13                      | 104                       | 0                         |
|                   | TNNR         | April  | 100           | 100            | 93                          | 67                       | 0.93                      | 0.67                      | 43                       | 0                        |
|                   |              | May    | 102           | 102            | 1.02                        | 0.81                      | 67                        | 0                         |
|                   |              | June   | 109           | 109            | 1.09                        | 0.66                      | 75                        | 0                         |
|                   |              | July   | 114           | 114            | 1.14                        | 0.76                      | 87                        | 0                         |
|                   |              | August | 123           | 123            | 1.23                        | 0.79                      | 101                       | 0                         |
|                   |              | September | 103     | 103            | 1.03                        | 0.65                      | 79                        | 0                         |
|                   |              | October | 96            | 96             | 0.96                        | 0.54                      | 33                        | 0                         |
|                   | WNNR         | April  | 100           | 100            | 0                           | 0                        | 0                         | 0                         |
|                   |              | May    | 0             | 0              | 0                           | 0                        | 0                         | 0                         |
|                   |              | June   | 0             | 0              | 0                           | 0                        | 0                         | 0                         |
|                   |              | July   | 0             | 0              | 0                           | 0                        | 0                         | 0                         |
|                   |              | August | 0             | 0              | 0                           | 0                        | 0                         | 0                         |
|                   |              | September | 0       | 0              | 0                           | 0                        | 0                         | 0                         |
|                   |              | October | 0             | 0              | 0                           | 0                        | 0                         | 0                         |
|                   | FTZNNR       | April  | 100           | 100            | 86                          | 45                       | 0.86                      | 0.45                      | 32                       | 0                        |
|                   |              | May    | 85            | 85             | 0.85                        | 0.33                      | 66                        | 0                         |
|                   |              | June   | 98            | 98             | 0.98                        | 0.75                      | 34                        | 0                         |
|                   |              | July   | 103           | 103            | 1.03                        | 0.88                      | 79                        | 0                         |
|                   |              | August | 106           | 106            | 1.06                        | 0.86                      | 98                        | 0                         |
|                   |              | September | 101     | 101            | 1.01                        | 0.76                      | 77                        | 0                         |
|                   |              | October | 91            | 23             | 0.91                        | 0.23                      | 34                        | 0                         |

Abbreviations: FNNR, Foping National Nature Reserve; TNNR, Tangjiahe National Nature Reserve; WNNR, Wolong National Nature Reserve; FTZNNR, Fengtongzhai National Nature Reserve; No. Fly Traps, mean the total number of traps placed at monitoring point; No. *P. okadai*, mean the total number of *Phortica okadai* collected in the 24h of sampling; Fly density (fly/cage /24h), mean the number of *Phortica okadai* per cage for 24h; No. infected *P. okadai*, mean the number of contain early stage of pre-infection larva of *Thelazia callipaeda* in fly haemocoel

Figures
Table 2 The density and infection situation of *Phortica okadai* in wildlife home range and surrounding villages, China, 2017

| Monitoring points | Region | Months | No. Fly Traps | No. *P. okadai* surrounding villages wildlife home range | Fly density (fly/cage /24h) surrounding villages wildlife home range | No. infected *P. okadai* surrounding villages wildlife home range |
|-------------------|--------|--------|---------------|--------------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| FNNR              | Shaanxi| April  | 100           | 181 81 1.81 0.81 90 0                                  |                                                 |                                                 |
|                   |        | May    | 221 88 2.21 0.88 121 0                               |                                                 |                                                 |
|                   |        | June   | 238 103 2.38 1.03 143 0                              |                                                 |                                                 |
|                   |        | July   | 241 113 2.41 1.13 213 0                              |                                                 |                                                 |
|                   |        | August | 272 129 2.72 1.29 222 0                              |                                                 |                                                 |
|                   |        | September | 203 109 2.03 1.09 107 0                          |                                                 |                                                 |
|                   |        | October | 180 99 1.80 0.99 111 0                             |                                                 |                                                 |
| TNNR              | Sichuan| April  | 100           | 103 78 1.03 0.78 52 0                                  |                                                 |                                                 |
|                   |        | May    | 116 86 1.16 0.86 56 0                                |                                                 |                                                 |
|                   |        | June   | 105 76 1.05 0.76 79 0                                |                                                 |                                                 |
|                   |        | July   | 124 72 1.24 0.72 94 0                                |                                                 |                                                 |
|                   |        | August | 113 89 1.13 0.89 79 0                                |                                                 |                                                 |
|                   |        | September | 97 71 0.97 0.71 59 0                             |                                                 |                                                 |
|                   |        | October | 92 62 0.92 0.62 31 0                                |                                                 |                                                 |
| WNNR              | Sichuan| April  | 100           | 0 0 0 0 0 0                                       |                                                 |                                                 |
|                   |        | May    | 0 0 0 0 0 0                                       |                                                 |                                                 |
|                   |        | June   | 0 0 0 0 0 0                                       |                                                 |                                                 |
|                   |        | July   | 0 0 0 0 0 0                                       |                                                 |                                                 |
|                   |        | August | 0 0 0 0 0 0                                       |                                                 |                                                 |
|                   |        | September | 0 0 0 0 0 0                             |                                                 |                                                 |
|                   |        | October | 0 0 0 0 0 0                                       |                                                 |                                                 |
| FTZNNR            | Sichuan| April  | 100           | 90 56 0.90 0.56 42 0                                 |                                                 |                                                 |
|                   |        | May    | 81 32 0.81 0.32 35 0                                 |                                                 |                                                 |
|                   |        | June   | 93 79 0.93 0.79 32 0                                 |                                                 |                                                 |
|                   |        | July   | 112 82 1.12 0.82 68 0                                |                                                 |                                                 |
|                   |        | August | 121 96 1.21 0.96 93 0                                |                                                 |                                                 |
|                   |        | September | 110 74 1.10 0.74 66 0                          |                                                 |                                                 |
|                   |        | October | 81 33 0.81 0.33 39 0                                |                                                 |                                                 |

**Abbreviations:** FNNR, Foping National Nature Reserve; TNNR, Tangjiahe National Nature Reserve; WNNR, Wolong National Nature Reserve; FTZNNR, Fengtongzhai National Nature Reserve; No. Fly Traps, mean the total number of traps placed at monitoring point; No. *P. okadai*, mean the total number of *Phortica okadai* collected in the 24h of sampling; Fly density (fly/cage /24h), mean the number of *Phortica okadai* per cage for 24h; No. infected *P. okadai*, mean the number of contain early stage of pre-infection larva of *Thelazia callipaeda* in fly haemocoe
| Monitoring points | Region   | Months | No. Fly Traps | No. P. okadai | Fly density (fly/cage /24h) | No. infected P. okadai |
|------------------|----------|--------|---------------|--------------|----------------------------|-----------------------|
|                  |          |        | surrounding villages | surrounding villages | surrounding villages | surrounding villages | surrounding villages |
| FNNR             | Shaanxi  | April  | 100           | 100          | 180           | 92        | 1.80        | 0.92        | 91           | 0         |
|                  |          | May    | 219           | 79           | 2.19          | 0.79      | 106         | 0          |
|                  |          | June   | 205           | 131          | 2.05          | 1.31      | 129         | 0          |
|                  |          | July   | 212           | 126          | 2.12          | 1.26      | 209         | 0          |
|                  |          | August | 284           | 123          | 2.84          | 1.23      | 223         | 0          |
|                  |          | September | 232       | 116          | 2.32          | 1.16      | 116         | 0          |
|                  |          | October | 149          | 83           | 1.49          | 0.83      | 109         | 0          |
| TNNR             | Sichuan  | April  | 100           | 100          | 108           | 61        | 1.08        | 0.61        | 64           | 0          |
|                  |          | May    | 121           | 71           | 1.21          | 0.71      | 63          | 0          |
|                  |          | June   | 112           | 82           | 1.12          | 0.82      | 73          | 0          |
|                  |          | July   | 132           | 89           | 1.32          | 0.89      | 85          | 0          |
|                  |          | August | 129           | 92           | 1.29          | 0.92      | 84          | 0          |
|                  |          | September | 82       | 83           | 0.82          | 0.83      | 67          | 0          |
|                  |          | October | 99           | 78           | 0.99          | 0.78      | 43          | 0          |
| WNNR             | Sichuan  | April  | 100           | 100          | 0             | 0         | 0           | 0          |
|                  |          | May    | 0             | 0            | 0             | 0         | 0           | 0          |
|                  |          | June   | 0             | 0            | 0             | 0         | 0           | 0          |
|                  |          | July   | 0             | 0            | 0             | 0         | 0           | 0          |
|                  |          | August | 0             | 0            | 0             | 0         | 0           | 0          |
|                  |          | September | 0       | 0            | 0             | 0         | 0           | 0          |
|                  |          | October | 0             | 0            | 0             | 0         | 0           | 0          |
| FTZNNR           | Sichuan  | April  | 100           | 100          | 53            | 31        | 0.53        | 0.31        | 26           | 0          |
|                  |          | May    | 83            | 53           | 0.83          | 0.53      | 42          | 0          |
|                  |          | June   | 62            | 56           | 0.62          | 0.56      | 33          | 0          |
|                  |          | July   | 124           | 72           | 1.24          | 0.72      | 71          | 0          |
|                  |          | August | 119           | 89           | 1.19          | 0.89      | 88          | 0          |
|                  |          | September | 102      | 88           | 1.02          | 0.88      | 78          | 0          |
|                  |          | October | 92            | 66           | 0.92          | 0.86      | 40          | 0          |

**Abbreviations:** FNNR, Foping National Nature Reserve; TNNR, Tangjiahe National Nature Reserve; WNNR, Wolong National Nature Reserve; FTZNNR, Fengtongzhai National Nature Reserve; No. Fly Traps, mean the total number of traps placed at monitoring point; No. P. okadai, mean the total number of *Phortica okadai* collected in the 24h of sampling; Fly density (fly/cage /24h), mean the number of *Phortica okadai* per cage for 24h; No. infected *P. okadai*, mean the number of contain early stage of pre-infection larva of Thelazia callipaeda in fly haemocoel.
Table 4 The density and infection situation of *Phortica okadai* in wildlife home range and surrounding villages, China, 2019

| Monitoring points | Region     | Months | No. Fly Traps | No. *P. okadai* | Fly density (fly/cage /24h) | No. infected *P. okadai* |
|-------------------|------------|--------|---------------|----------------|----------------------------|-------------------------|
|                   |            |        | surrounding villages | surrounding villages | surrounding villages | surrounding villages | surrounding villages | surrounding villages |
| FNNR              | Shaanxi    | April  | 100           | 100            | 198             | 197                     | 1.98                   | 1.97                 | 101                  | 115                  |
|                   |            | May    |               |                | 201             | 276                     | 2.01                   | 2.76                 | 166                  | 185                  |
|                   |            | June   |               |                | 225             | 224                     | 2.25                   | 2.24                 | 186                  | 166                  |
|                   |            | July   |               |                | 278             | 256                     | 2.78                   | 2.56                 | 212                  | 206                  |
|                   |            | August |               |                | 266             | 299                     | 2.66                   | 2.99                 | 202                  | 218                  |
|                   |            | September |            |                | 223             | 245                     | 2.23                   | 2.45                 | 167                  | 191                  |
|                   |            | October |               |                | 187             | 198                     | 1.87                   | 1.98                 | 101                  | 142                  |
| TNNR              | Sichuan    | April  | 100           | 100            | 123             | 44                      | 1.23                   | 0.44                 | 67                   | 0                    |
|                   |            | May    |               |                | 102             | 82                      | 1.02                   | 0.82                 | 55                   | 0                    |
|                   |            | June   |               |                | 101             | 99                      | 1.01                   | 0.99                 | 72                   | 0                    |
|                   |            | July   |               |                | 115             | 83                      | 1.15                   | 0.83                 | 71                   | 0                    |
|                   |            | August |               |                | 113             | 91                      | 1.13                   | 0.91                 | 78                   | 0                    |
|                   |            | September |            |                | 79              | 74                      | 0.79                   | 0.74                 | 41                   | 0                    |
|                   |            | October |               |                | 87              | 76                      | 0.87                   | 0.76                 | 59                   | 0                    |
| WNNR              | Sichuan    | April  | 100           | 100            | 0               | 0                       | 0                      | 0                    | 0                    | 0                    |
|                   |            | May    |               |                | 0               | 0                       | 0                      | 0                    | 0                    | 0                    |
|                   |            | June   |               |                | 0               | 0                       | 0                      | 0                    | 0                    | 0                    |
|                   |            | July   |               |                | 0               | 0                       | 0                      | 0                    | 0                    | 0                    |
|                   |            | August |               |                | 0               | 0                       | 0                      | 0                    | 0                    | 0                    |
|                   |            | September |            |                | 0               | 0                       | 0                      | 0                    | 0                    | 0                    |
|                   |            | October |               |                | 0               | 0                       | 0                      | 0                    | 0                    | 0                    |
| FTZNNR            | Sichuan    | April  | 100           | 100            | 61              | 49                      | 0.61                   | 0.49                 | 39                   | 0                    |
|                   |            | May    |               |                | 78              | 67                      | 0.78                   | 0.67                 | 48                   | 0                    |
|                   |            | June   |               |                | 59              | 48                      | 0.59                   | 0.48                 | 46                   | 0                    |
|                   |            | July   |               |                | 109             | 84                      | 1.09                   | 0.84                 | 62                   | 0                    |
|                   |            | August |               |                | 106             | 83                      | 1.06                   | 0.83                 | 64                   | 0                    |
|                   |            | September |            |                | 99              | 52                      | 0.99                   | 0.52                 | 32                   | 0                    |
|                   |            | October |               |                | 88              | 51                      | 0.88                   | 0.51                 | 27                   | 0                    |

**Abbreviations:** FNNR, Foping National Nature Reserve; TNNR, Tangjiahe National Nature Reserve; WNNR, Wolong National Nature Reserve; FTZNNR, Fengtongzhai National Nature Reserve; No. Fly Traps, mean the total number of traps placed at monitoring point; No. *P. okadai*, mean the total number of *Phortica okadai* collected in the 24h of sampling; Fly density (fly/cage /24h), mean the number of *Phortica okadai* per cage for 24h; No. infected *P. okadai*, mean the number of contain early stage of pre-infection larva of *Thelazia callipeda* in fly haemocoel
Figure 1

The wildlife nature reserves in which the monitoring points of Phortica okadai and wildlife were set up, China, 2016-2019. Abbreviations: FNNR, Foping National Nature Reserve; TNNR, Tangjiahe National Nature Reserve; WNNR, Wolong National Nature Reserve; FTZNNR, Fengtongzhai National Nature Reserve Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.
Figure 2

Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Figure 3

a The early stage of pre-infection larva of Thelazia callipaeda in haemocoel of Phortica okadai; b Thelazia callipaeda with the buccal capsule, pharynx, and esophagus of the anterior segment of the worm body and its serrated, wrinkled surface (arrow); c The caudal end of a worm; d Larvae in the female’s uterus (arrow)
Figure 4

PCR amplified electrophoresis map of cox1 gene of Thelazia callipaeda. Abbreviations: M, Marker; 1, cox1 gene positive products of Thelazia callipaeda from wild giant panda; 2, cox1 gene positive products of Thelazia callipaeda from wild boar; 3, cox1 gene positive products of Thelazia callipaeda from leopard cat; 4, cox1 gene positive products of Thelazia callipaeda from black bear

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