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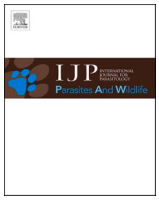

Studies on the embryonic development and larval infection potential of the stomach bot flies, *Gasterophilus pecorum*

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ABSTRACT

Endangered Przewalski's horses have faced severe infections from *Gasterophilus pecorum* (Diptera, Gastrophilidae) in Xinjiang's Kalamaili Nature Reserve (KNR). This study examines *G. pecorum*'s development and infection patterns in embryonic and larval stages, crucial for understanding horse botfly disease in desert grasslands. For the incubation of *G. pecorum* fertilized eggs, we established the six distinct temperature gradients: 16 °C, 20 °C, 24 °C, 28 °C, 30 °C, and 32 °C. Using the least squares method, we calculated the correlation between the developmental threshold temperature of the eggs and their cumulative effective temperature. Furthermore, we meticulously recorded the survival duration of the larvae across a spectrum of temperature gradients (−20 °C, −10 °C, 4 °C, 10 °C, 20 °C, and 30 °C) and under varying conditions (dark and light). This method allows us to analyze and interpret the impact of these environmental factors on larval survival durations. 1) The formula for predicting the embryonic development period of *G. pecorum* was $N = (182.7 \pm 12.03)/[T-(3.191 \pm 1.48)]$, where the developmental threshold temperature was 3.191 ± 1.48 °C, and the effective accumulated temperature was 182.7 ± 12.03 d°C 2) The model describing the relationship between the embryonic development rate and temperature was: $y = 0.0001x^2+0.0007x+0.0378$, demonstrating a positive correlation between the development rate and temperature (R-sq = 0.989, $p < 0.001$). 3) Larvae in the dark group exhibited a longer survival time, with the longest being 9 months at 4 °C. The adaptation of *G. pecorum*'s embryonic development to cold temperature, combined with the extended survival period of larvae in the egg state, significantly increases the infection potential of *G. pecorum* in colder climates. This discovery offers essential insights into the predominance of *G. pecorum* in the KNR region and provides a crucial biological basis for the prevention of myiasis and the conservation of vulnerable species, such as Przewalski's horses.

1. Introduction

Gasterophilus pecorum myiasis is prevalent among equine populations in the Kalamaili Nature Reserve (KNR), Xinjiang (Liu et al., 2016; Huang et al., 2022). Remarkably, within the reintroduced population of *Equus przewalskii*, the infection rate by *G. pecorum* reaches an alarming 100%, thereby posing a significant threat to the reintroduction efforts of Przewalski's horses (Huang et al., 2022). The overwhelming infection intensity of *G. pecorum*, with an average of 1875 flies per horse, and its high prevalence, accounting for 98.51% of the infections, establish it as the predominant species in *Gasterophilus* in the KNR (Huang et al., 2016). This predominance contrasts with the global prevalence of *G. intestinalis* and *G. nasalis* as the primary botfly species in other regions (Pandey et al., 1992; Agneessens et al., 1998; Lyons et al., 1994; Mukbel et al., 2001).

G. pecorum, a member of the Gasterophilidae family within the order Diptera, is exclusively parasitic in its larval stage in the digestive tract of equines. It absorbs nutrients from its host and develops from first instar larvae to third instar maturity, while secreting toxins that can lead to

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Abbreviations

KNR Kalamaili Nature Reserve

anemia, emaciation, and in severe cases, exhaustion and death of the host (Czosnek, 1998; Smith et al., 2005; Pawlas et al., 2007). In natural environments, the fertilized eggs of *G. pecorum* hatch into first instar larvae within approximately one week (Colwell, 2006). These larvae stay within the eggshell, ready to be ingested by a host to initiate infection. Initially parasitizing the host's mouth, they develop into second instar larvae in about one month, and then move to the gastrointestinal tract, where they mature third instar larvae over a period of eight to nine months (Cogley, 1991). *G. pecorum* exhibits a notably higher egg-laying capacity, with a range of 1300 to 2425 eggs, exceeding that of other species within the same genus (Zumpt, 1965). In the KNR, the average number of eggs laid in a single *Spita caucasica* by *G. pecorum* was 3.6 (Zhang et al., 2021; Huang et al., 2021), and the third instar mature larvae of *G. pecorum* have two peaks, specifically in April and August (Zhang et al., 2021). Unlike other species of the same genus, which lay their eggs on the surface of equine animals, *G. pecorum* lays its eggs on specific plants (Colwell, 2006; Zhou et al., 2020).

As a reintroduced species, the Przewalski's horse was released into the desert steppe in the KNR in 2001, and has since attracted continuous attention of people (Xia et al., 2014). Our research team, while monitoring their adaptation, discovered a severe infection of *G. pecorum* in these horses (Huang et al., 2022). Autopsies of Przewalski's horses that died accidently in winter found by chance that the first instar larvae of *G. pecorum* were parasitized in the oral cavity, suggesting the capability of *G. pecorum* to infect at low-temperature. Up to now, there have been no reports detailing the embryonic development and the survival ability of *G. pecorum* at low-temperature. To elucidate the epidemiology of *G. pecorum* and analyze the phenomenon of wild equines being invaded by botfly eggs in winter, we conducted studies on the embryonic development of *G. pecorum* eggs and the survival ability of larvae in the egg state. This research aims to expand the understanding of *G. pecorum*'s life cycle and provide a theoretical basis for the precise prediction and control of myiasis in wild equids.

2. Methods

2.1. Study area

The KNR (40°36′–46°00′ N, 88°30′–90°03′ E) is located in the Junggar Basin in Xinjiang, China, covering an area of 14,856.48 km^2 (Zhang et al., 2023). Characterized as a desert steppe, the region has an average annual temperature of 2.4 °C. In recent years, January, the coldest month, has experienced an average temperature below −20 °C, with the lowest being −38 °C; conversely, July, the hottest month, has seen the average temperature above 25 °C, with peak temperatures soaring up to 50 °C (Zhang et al., 2015, 2023). The annual rainfall is approximately 160 mm, significantly lower than the annual evaporation of approximately 2000 mm (Zhang et al., 2015). The reserve serves as a sanctuary for diverse wildlife, including the Przewalski's horse, the Mongolian wild donkey, and the goose-throated antelope (Xia et al., 2014). The vegetation in the reserve is relatively simple and sparse, generally covering 20–30% of the area. The predominant plant species include *S. caucasica*, *Ceratoides latens*, *Artemisia* spp., and *Convolvulus tragacanthoides* Turcz. (Zhou et al., 2020; Zang et al., 2017).

2.2. Experiment materials

From April to May 2018, we tracked Przewalski's horses and observed its defecation behavior in the reserve. During this period, we collected third instar mature larvae that were naturally excreted in the fresh feces (≤5min), with reference to the third instar mature larvae identification method of *Gasterophilus* (Zumpt, 1965; Zhang et al., 2023; Li et al., 2019). The larvae collected in the field were subsequently brought indoors for cultivation. Transparent plastic cups (9.3 cm top diameter, 6.2 cm bottom diameter, 6.5 cm height) were used as incubation containers for pupation, each filled with 5 cm of fine sand soil (Zhang et al., 2023). The third instar mature larvae were placed on this sand soil for their development. The development of the pupae was observed and recorded until their emergence as adult bot flies. Subsequently, female and male bot flies were placed in 40 cm × 30 cm × 20 cm cages for natural mating. The fertilized eggs were then collected for experimental purposes.

2.3. Experiment devices

The study used transparent culture containers and electronic thermometers for measurements. To maintain controlled environmental conditions, we employed a PRX-450B artificial climate chamber (temperature error ± 1 °C, Ningbo Saifu Experimental Instrument Co., Ltd.) and an HRX-1200 low-temperature incubator (temperature error ± 1 °C, Changzhou Henglong Instrument Co., Ltd.). Additional equipment included acrylic insect cages, insect dissection tweezers ST-17 and 3-SA (Shanghai Wettus Tools Co., Ltd.), and an SZ51 stereomicroscope equipped with LED lighting/SZ2-ILST (Olympus corporation, Tokyo, Japan).

2.4. Embryonic development

The fertilized eggs were incubated under six temperature gradients:16 °C, 20 °C, 24 °C, 28 °C, 30 °C, and 32 °C, with a relative humidity maintained at 30% ± 2% RH and a photoperiod of L: D = 14:10 (12,000 lx). Each temperature gradient was replicated in three separate trials. The development of the embryos was examined and recorded every 12 h using a stereoscopic microscope. This process involved a destructive examination process, where 10 eggs from each group were randomly selected for inspection until the eggs in each group reached maturity.

2.5. Egg-state larval survival period

Following the study of embryo development, the mature egg-state larvae were placed in Petri dishes. The fertilized eggs were incubated in four temperature gradient groups (4 °C, 10 °C, 20 °C, 30 °C) with a relative humidity maintained at 30% ± 2% RH and a photoperiod of L: D = 14:10 (12,000 lx), under both light and dark conditions (the latter achieved by wrapping in tin foil). Additionally, a survival period testing for egg-state larvae was conducted at −10 °C and −20 °C under dark conditions. The survival of these larvae was routinely checked every 10 days, involving a random examination of the number of viable eggs in each group. If the survival rate of the eggs under examination was determined to be 0%, it was reassessed after an additional 10 days.

2.6. Embryo development analysis

The formula of effective accumulated temperature is as follows:

$$K = N(T - C) \quad (1)$$

In formula (1), K is the effective accumulated temperature, N is the development period, T is the test temperature, and C is the development threshold temperature. Since N is the inverse of the developmental rate V, the formula can be re-expressed as follows:

$$T = C + KV \quad (2)$$

Formula (2) represents a linear regression equation between the test

temperature (T) and the development rate (V). In this linear relationship, the effective accumulated temperature (K) and the development threshold temperature (C) are the undetermined coefficients. Combining the developmental periods measured under six treatment temperatures, we calculated the developmental threshold temperature (C) and the effective accumulated temperature (K) for the eggs of *G. pecorum* using the least square method, along with their respective standard errors: the standard error of the developmental threshold temperature (Sc) and the standard error of the effective accumulated temperature (Sk) (Wang et al., 2015; Zhang et al., 2023). The calculation formulas are as follows:

$$C = \frac{\sum V^2 \sum T - \sum V \sum VT}{n\sum V^2 - (\sum V)^2} \quad (3)$$

$$K = \frac{n\sum VT - \sum V \sum T}{n\sum V^2 - (\sum V)^2} \quad (4)$$

According to the effective accumulated temperature rule, the theoretical value of temperature is T′, T' = V + CK.

$$Sc = \sqrt{\frac{\sum (T - T')^2 \left(\frac{1}{n} + \frac{\overline{V}^2}{\sum (V - \overline{V})^2}\right)}{n - 2}} \quad (5)$$

$$S_k = \sqrt{\frac{\sum (T - T')^2}{(n - 2)\sum (V - \overline{V})^2}} \quad (6)$$

In formulas (3)-(6), n is the number of temperature gradients, T is the experimental temperature (°C), V is the development rate (1/N), N is the development period (d), T′ is the theoretical temperature value, and $\overline{V}$ is the mean value of the development rate.

According to the development threshold temperature (C) and the effective accumulated temperature (K), a linear regression equation between the development rate (V) and the test temperature (T) was established. The significance of the regression model was tested using SPSS 26.0 to establish a formula to predict the developmental period:

$$N = \frac{K \pm S_k}{T - (C \pm S_c)} \quad (7)$$

2.7. Climate database

Monthly mean temperature data spanning from 2011 to 2020 were collected from the nearest meteorological station (45°22′ N, 90°32′ E) in the KNR (http://data.cma.cn). We then calculated the mean and standard deviation of the monthly temperatures over this ten-year period.

2.8. Data analysis

The model fit for the rate of embryo development at various temperatures was established using the curve regression analysis module, which performed multiple regression analyses. The most suitable regression equation was established based on the analysis results. The significant effects of different temperature and light conditions on larval survival were explored using Two-way ANOVA, with $P < 0.05$ indicating a significant difference. Data analysis was performed with SPSS 26.0, and figures were generated using SigmaPlot 12.0.

3. Results

3.1. Embryonic development duration of G. pecorum

Observations revealed that the embryo is in a liquid state initially inside the egg. Within a development span of 3–4 days, the embryo starts to manifest distinct characteristics, including the formation of mouthhooks and spines, marking its development into the first instar larvae stage.

The duration of embryonic development of *G. pecorum* at various temperature conditions was analyzed using formulas (1) to (7) to ascertain the developmental threshold temperature and the effective accumulated temperature (Table 1). At temperatures of 16 °C, 20 °C, 24 °C, 28 °C, 30 °C, and 32 °C, the incubation periods for the eggs were recorded as 14.00, 10.33, 9.17, 7.67, 6.83, and 6.17 days, respectively. A notable trend was observed where the embryonic development period of *G. pecorum* inversely correlated with the increasing temperatures. The predictive formula for the embryonic development period of *G. pecorum* was: N = (182.7 ± 12.03)/[T-(3.191 ± 1.48)]. The developmental threshold temperature was 3.191 ± 1.48 °C, and the effective accumulated temperature was calculated to be 182.7 ± 12.03 d°C.

3.2. The relationship between temperature and the embryonic development rate of G. pecorum

The relationship between temperature and the development rate of *G. pecorum* eggs was graphically represented with the developmental periods as the ordinate and the temperature gradient as the abscissa. Linear, polynomial, and exponential regression models were all applicable to describe the relationship between the developmental rate of *G. pecorum* eggs and temperature. However, the polynomial model provided a more accurate depiction of this relationship (Fig. 1). Across the six temperature gradients of 16 °C, 20 °C, 24 °C, 28 °C, 30 °C, and 32 °C, a positive correlation was observed between temperature and the developmental rate of *G. pecorum* eggs. With increasing temperature, the developmental rate of *G. pecorum* eggs was significantly accelerated. Regression analysis indicated that the polynomial curve model is: $y = 0.0001x^2+0.0007x+0.0378$ (R-sq = 0.989, $P < 0.001$), demonstrated the highest degree of fit.

3.3. Survival period of egg-state larvae population of G. pecorum

Based on the results of the embryonic development experiments, we observed the survival period of the egg-state larvae under various conditions after they had matured. The surviving larvae in the egg state was characterized by their transparent body and a distinctly visible, light-yellow digestive tract. They appeared plump, well-hydrated, and exhibited writhing movements upon breaking out of the eggshell. In contrast, the dead larvae in the egg state presented a shriveled appearance, predominantly brownish-yellow in color, and exhibited severe dehydration.

Throughout the duration of the experiment, a total of 2980 eggs were dissected for analysis. The survival time of the larvae exhibited notable variations under different experimental conditions. The average survival times were observed in the following order: 4 °C dark >4 °C light >10 °C dark >10 °C light >20 °C dark >20 °C light >30 °C light >30 °C light > −10 °C dark > −20 °C dark. Notably, the larvae in the 4 °C dark group demonstrated the longest survival, reaching up to 270 days, with an average survival time of 219.0 ± 46.1 days. However, under extremely low-temperature conditions, such as in the −20 °C dark group, the larvae showed the shortest survival period, lasting only up to 50 days, with an average survival time of 31.0 ± 10.9 days (Table 2). Furthermore, at the identical temperature, the factor of light exposure significantly affected the larvae's survival time, with observations indicating a notably longer survival in dark environments (Fig. 2).

The results of Two-way ANOVA analysis demonstrated that the survival duration of egg-state larvae was significantly affected by both temperature and light ($F = 868.121$, $P < 0.001$; $F = 108.218$, $P < 0.001$). Moreover, the interaction between temperature and light, under various treatment conditions, also significantly affected the survival period of egg-state larvae ($F = 33.636$, $P < 0.001$) (Table 3).

Table 1
The embryonic development threshold temperature and the effective accumulated temperature of *G. pecorum*.

	T(°C)	N(d)	V = 1/N	VT	V^2	T'	(T-T')	$(T-T')^2$	$(V-\overline{V})$	$(V-\overline{V})^2$
1	16	14.000	0.071	1.143	0.005	16.240	−0.240	0.058	−0.048	0.0023
2	20	10.333	0.096	1.935	0.009	20.876	−0.876	0.768	−0.023	0.0005
3	24	9.167	0.109	2.618	0.012	23.113	0.886	0.786	−0.010	0.0001
4	28	7.667	0.130	3.625	0.017	27.010	0.990	0.980	0.011	0.0001
5	30	6.833	0.146	4.390	0.021	29.940	0.060	0.004	0.027	0.0007
6	32	6.167	0.162	5.189	0.026	32.801	−0.801	0.642	0.043	0.0018

Note: T: Test temperature; N: Development duration; V: Development rate; $\overline{V}$: Average development rate; T': Theoretical temperature value, T' = C + KV.

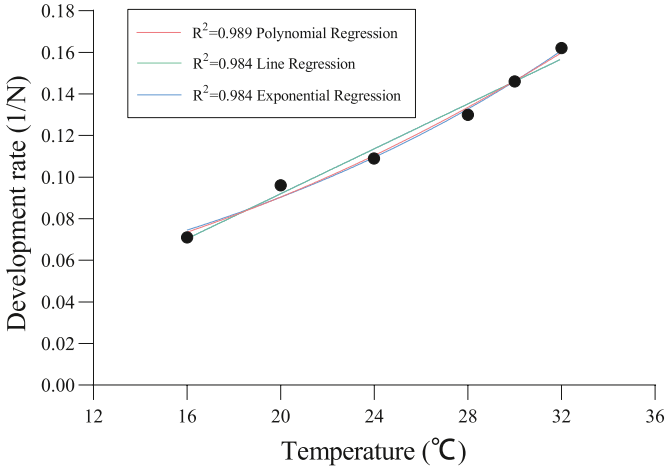

Fig. 1. The relationship between the embryonic development period and the temperature of *G. pecorum*.

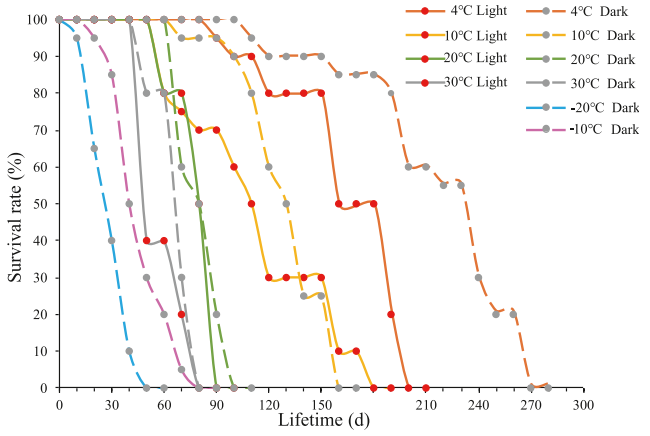

Fig. 2. Larval survival period of *G. pecorum*.

Table 2
The average survival time of the *G. pecorum* larvae under different conditions.

Condition		Average survival time ± SD (d)
4 °C	Dark	219.0 ± 46.1
	Light	166.5 ± 33.8
10 °C	Dark	131.5 ± 23.2
	Light	114.5 ± 40.5
20 °C	Dark	83.0 ± 11.9
	Light	81.0 ± 11.4
30 °C	Dark	69.0 ± 10.5
	Light	60.0 ± 12.7
−10 °C	Dark	48.5 ± 15.3
−20 °C	Dark	31.0 ± 10.9

Table 3
The effects of light and temperature on the development and lifetime of *G. pecorum* larvae.

Condition	Lifetime	
	F	P
Light	868.121	<0.001***
Temperature	108.218	<0.001***
Light and Temperature	33.636	<0.001***

3.4. Prediction of the egg-state larvae population of G. pecorum

In our research, we examined the survival duration of the egg-state larvae of *G. pecorum* across various temperatures (Fig. 3a). Furthermore, we correlated the patterns of its three life stages (third instar larvae, pupa, and adult) throughout the year in the KNR (Zhang et al., 2021) with the monthly temperature fluctuations of the locale (Fig. 3b). This comprehensive analysis facilitated the projection of the yearly occurrence pattern of *G. pecorum*'s egg-state larvae in the KNR (Fig. 3c). The results indicate that the epidemic period of *G. pecorum*'s egg state larvae spans 9 months annually, mirroring the lifecycle patterns of both larvae and adults, which are marked by a bimodal distribution. Specifically, there are two critical periods in a year (May ~ June and August ~ September), when local equids were at high risk of infection.

4. Discussion

The eggs of *G. pecorum* demonstrate remarkable resilience in low-temperature conditions, as indicated by their developmental threshold temperature of 3.191 ± 1.48 °C and an effective accumulated temperature of 182.7 ± 12.03 d°C. This developmental threshold temperature was notably lower compared to those of other parasitic flies, as documented by Catts (1967) and Pruett and Kunza (1996). Notably, parasitic flies from the different genus of the same family, such as *Hypodermatinae tarandi* and *Hypoderma lineatum*, also demonstrated similar adaptive characteristics (Pruett and Kunz, 1996; Karter et al., 1992). In contrast, the developmental threshold temperature of *G.intestinalis*, which shared its habitat with *G. pecorum*, was reported to be around 10 °C (Sukhapesna et al., 1975), indicating a less pronounced adaptation to lower temperatures (Gilbert and Raworth, 1996; Zhang et al., 2023). Furthermore, within the temperature range of 16 °C–30 °C, the embryonic development rate of *G. intestinalis* exceeded that of *G. pecorum* (Zhang et al., 2023). The lower developmental threshold temperature of *G. pecorum* contributes to its greater developmental potential, which may have been a pivotal factor contributing to the high infection rates of *G. pecorum* observed in the KNR.

Our study found that the survival time of *G. pecorum* egg state larvae varies with temperature. The survival time of larvae was the longest at 4 °C, and with the increase of temperature to 10 °C, 20 °C, and 30 °C, their survival time correspondingly decreased. This biological phenomenon observed in *G. pecorum* may be attributed to its metabolic

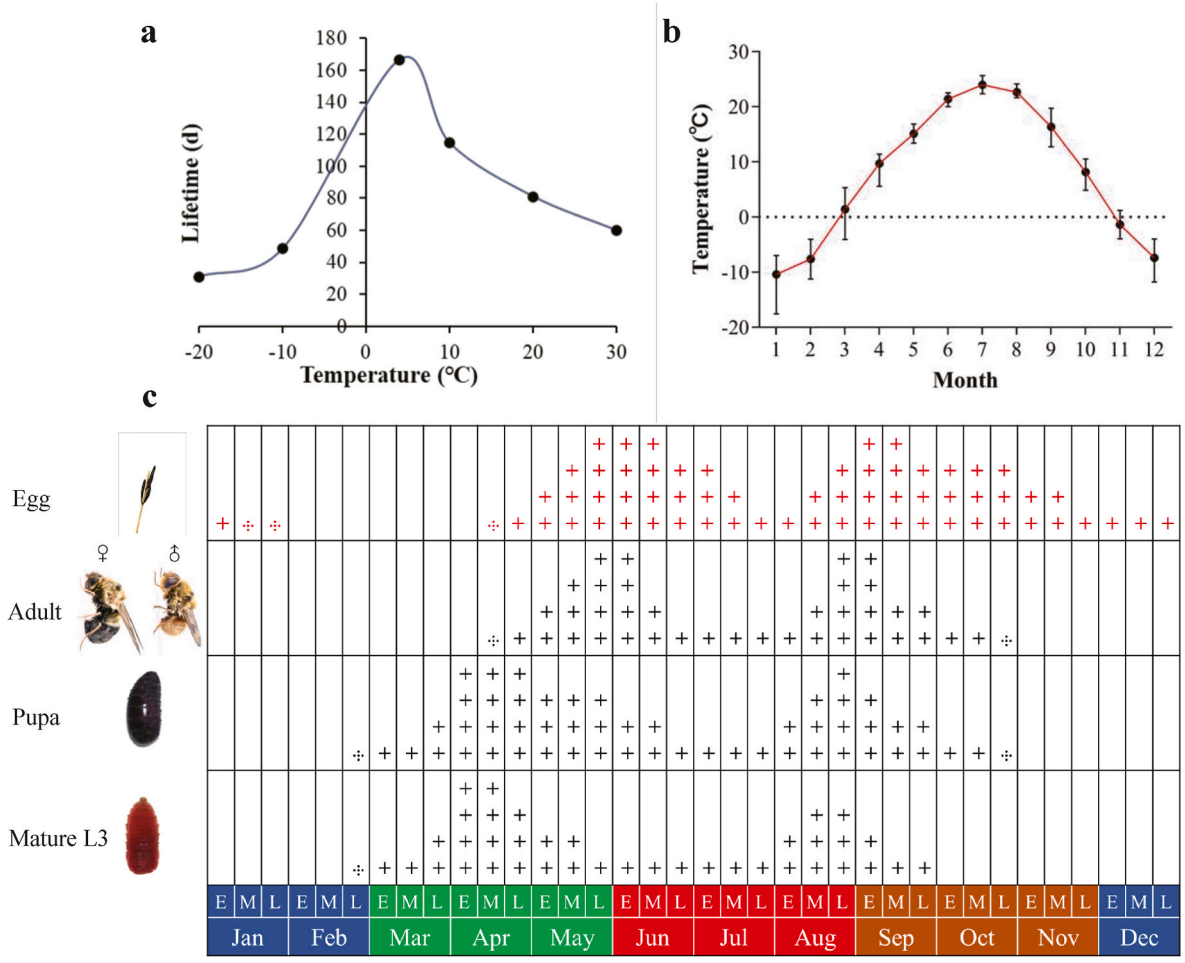

Fig. 3. Life history of *G. pecorum* and prediction of egg state larvae population. Note: The survival period of egg state larvae of G. pecorum at different temperatures (a); The monthly average temperatures in Kalamaili nature reserve (KNR) in recent ten years (b); Changes in parasite population in vitro of the host (c), +: mild; ++: moderate; +++: considerable; ++++: severe; ⁘: potential.

activity levels (Gilbert and Raworth, 1996). At lower temperatures, the metabolic rate of insects slows down (Gilbert and Raworth, 1996; Renault et al., 2002), thus reducing the energy expenditure necessary for egg development in the *G. pecorum*. However, an interesting pattern emerged at sub-zero temperatures, such as −10 °C and −20 °C, where larval survival with increasing temperatures. This could be due to the near cessation of life activities under extreme low-temperatures (Renault et al., 2002). Therefore, this study speculates that a slight increase in temperature may provide sufficient energy, thereby extending the survival time of *G. pecorum* larvae. These findings highlight the adaptability of the *G. pecorum* to temperature fluctuations and its survival strategy in coping with complex external environmental conditions. Comparison of related studies revealed that the larvae of the genus *Gasterophilus* are highly adaptive to low-temperature. For instance, egg-state larvae of the *G. nigricornis* have been documented to survive up to 140 days in low-temperature conditions (Zumpt, 1965), while *G. nasalis* larvae can survive for as long as 115 days (Enileeva, 1987). In addition, the study by Zumpt (1965) also revealed that in warmer climates (Zumpt, 1965), the survival duration of *G. pecorum* larvae was notably reduced as temperatures increased. This finding aligns with the observations of *G. pecorum* made in the current study.

The impact of varies light conditions on the survivability of egg-state larvae was also examined. Under laboratory conditions, it was observed that egg-state larvae exposed to light had a reduced survival period compared to those maintained in dark conditions. This disparity is likely due to the hastened metabolic rate in larvae subjected to light, resulting in greater water loss (Zumpt, 1965). These insights contribute to our understanding of *G. pecorum*'s adaptation to low-temperature environments. The larvae exhibited an extended survival period under cold and dark conditions, conferring a survival advantage in their natural habitats. Understanding the adaptive strategies of *G. pecorum* eggs is pivotal for elucidating their behavior and distribution in nature.

The low developmental threshold temperature and the extended egg viability of *G. pecorum* indicate their potential for year-round activity. This characteristic increases their survival and reproductive capabilities across various geographical regions and climates. *G. pecorum* is predominantly found in mid to high latitudes, as evidenced by its distribution in northern Xinjiang (44–46°N) and central Inner Mongolia (41–42°N) of China (Wang et al., 2016), Mongolia (47°N) (Dorzh and Minár, 1971), northern and central of Kazakhstan (47–54°N) (Ibrayev et al., 2015), Yakutia Republic of Russia (55–76°N) (Reshetnikov et al., 2014), southern Italy (39–41°N) (Ortanto et al., 2005), northern Iran (36–37°N) (Zaheri et al., 2015), among other regions. In particular, *G. pecorum* is an absolute predominant species in the mid to high latitudes in the KNR (Huang et al., 2021). These regions are characterized by significant temperature fluctuations, where *G. pecorum* leverages its low-temperature tolerance to maintain survival and reproduction, thereby increasing the risk of severe infections in specific regions.

The *G. pecorum* was the only *Gasterophilus* spp. that oviposited on grass (Chereshnev, 1951; Zumpt 1965), and also laid eggs on *Stipa caucasica* in Xinjiang, China (Liu et al., 2016; Zhou et al., 2020). The *G. pecorum* eggs embryonic development complete, the larvae remain in

the eggshell and hatch only when the plants are taken by hosts (Zupmt, 1965; Huang et al., 2021). The larvae are very resistant to adverse conditions and can remain alive in the egg-shells for many months (Zumpt, 1965). The duration of larval survival within the eggshell dictates the threat period to the equids. Our research has revealed that the *G. pecorum* larval stages remain viable across a wide temperature range, from 4 °C to 30 °C, for several months. Specifically, at 30 °C with light exposure, larvae exhibit an average survival duration of 60 days. In contrast, in darkness at 4 °C, their survival can extend up to 270 days, and at −20 °C in darkness, they can survive for as long as 50 days. The growth pattern of *S. caucasica* in the KNR, with peak growth in May and June and a regreening phenomenon in September (Huang et al., 2021; Zhang et al., 2021), aligns with the predicted survival periods of the *G. pecorum* larvae identified in our study. The robust resistance of the *G. pecorum* larval stages to unfavorable conditions enables their persistence in the wild for several months, indicating that the risk of the *G. pecorum* infestation in equids extends over most of the year, posing a prolonged threat to equine health.

The low-temperature conditions in the KNR during spring create an ideal environment for the survival of *G. pecorum*, resulting in persistent and severe infections among equine species. The larvae exhibit development at low-temperatures and extended survival times, with two peak periods observed in April and August in the field (Zhang et al., 2021). This indicates that the larvae are particularly aggressive in this ecological environment, posing a serious threat to Przewalski's horses. These findings are critical to understanding the spread and control of the *G. pecorum* in the KNR. The prolonged infection of Przewalski's horses with *G. pecorum* has not only contributed to a decline in their population but may also impacted the ecological balance in the KNR. Consequently, our study not only improves the understanding of the biology of *G. pecorum*, but also has important implications for developing effective management and conservation strategies.

5. Conclusions

The study firstly represents a detailed investigation into the embryonic development and low-temperature survival capabilities of *G. pecorum*. The findings indicate that *G. pecorum* exhibits high adaptability to the cool spring conditions in arid desert steppes, with a notably low embryonic developmental threshold temperature. This characteristic enables them to survive and reproduce under extreme conditions. By analyzing the embryonic development rate of *G. pecorum* at various temperatures, a significant positive correlation was observed between temperature and the development rate. This correlation is crucial for understanding the ecological adaptation mechanism of *G. pecorum* to temperature fluctuations and for predicting its survival and reproductive capacities under diverse climates. Moreover, the ability of *G. pecorum* to survive long term at low-temperature and in dark conditions significantly increases their potential to infect hosts. For instance, the larvae in the dark group at 4 °C exhibited the longest survival time, reaching up to 270 days, which poses a further potential threat to the Przewalski's horse. Therefore, this study not only provides crucial information for understanding the biological characteristics of *G. pecorum*, but also lays a theoretical foundation for the development of ecological prevention and control strategies against myiasis. This is particularly significant in evaluating the mechanism of severe infection of Przewalski's horses.

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Authors' contributions

Yu Zhang: Data analysis, writing and modification; Ke Zhang: Data analysis and writing; Other authors: Contribute to data collection and review the manuscript; Kai Li and Heqing Huang: Check and review the manuscript.

Ethics approval and consent to participate

The study was performed in accordance with the relevant guidelines and regulations regarding animal welfare. All experimental protocols were approved by the Ethic and Animal Welfare Committee, Beijing Forestry University.

Consent for publication

Not applicable.

Declaration of interest statement

Title of Manuscript: Studies on the Embryonic Development and Larval Infection Potential of the stomach bot flies, *Gasterophilus pecorum*.

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