German Journal of Veterinary Research

eISSN:2703-1322

Research article

First molecular characterization and distribution of Oestrid fly larvae (Diptera: *Oestridae*) infesting horses and camels in Kazakhstan

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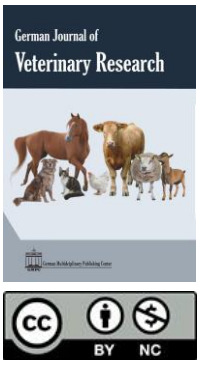

Article History:
Received: 05-Aug-2026
Accepted: 02-Oct-2026
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Abstract

The prevalence, species composition, seasonal dynamics, and molecular characteristics of oestrid bot flies infesting camels and horses were investigated in the Atyrau and Kyzylorda oblasts of Kazakhstan. In total, 169 animals were examined between 2024 and 2025. *Cephalopina titillator* larvae were identified in camels, while *Gasterophilus intestinalis* and *G. pecorum* larvae were detected in horses. Adult *G. intestinalis* and *Rhinoestrus usbekistanicus* were also collected during field surveys. The prevalence of *C. titillator* infestation in camels was 55.0% in 2024 and 52.1% in 2025. A pronounced seasonal pattern was observed, with prevalence reaching 75.8% in June in Atyrau Oblast and 75.0% in July in Kyzylorda Oblast, followed by a marked decline by September. Neither geographic region nor year was significantly associated with infestation prevalence. Rhinoestrosis was detected in 11.8% of examined horses in 2024 and 5.8% in 2025. Gasterophilosis caused by *G. intestinalis* was detected in all 10 horses examined in Atyrau Oblast during targeted examinations. Partial mitochondrial *cox1* sequences corroborated the morphological identification of *C. titillator*, *G. intestinalis*, and *G. pecorum*, with the sequences clustering with conspecific reference sequences in phylogenetic analysis. This study presents the first molecular analysis of bot flies infesting horses and camels in Kazakhstan, the first molecular confirmation of *R. usbekistanicus* in western Kazakhstan, and documents the recurrence of aberrant localization of *G. pecorum* larvae more than 40 years after its initial report. These findings expand current knowledge regarding the diversity, distribution, seasonal dynamics, and molecular characteristics of oestrid bot flies in Kazakhstan and provide a foundation for continued surveillance and integrated morphological and molecular investigations.

Keywords: *Cephalopina titillator*, *Rhinoestrus usbekistanicus*, *Gasterophilus intestinalis*, *Gasterophilus pecorum*, West Kazakhstan

Citation: Kenessary, S., Zhaksylykova, A., Sayakova, Z., Abdibaeva, A., Aryssova, E., Kidiraliyev, E., Tulepov, B., Turkeyev, M., Abdybekova, A., Kiyan, V., Jazina, K., and Uakhit, R. 2026. First molecular characterization and distribution of oestrid fly larvae (*Diptera: Oestridae*) infesting horses and camels in Kazakhstan. Ger. J. Vet. Res. 6 (4): 11-24. https://doi.org/10.51585/gjvr.2026.4.0207

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Introduction

Oestrid flies (Diptera: *Oestridae*) are obligate mammalian parasites whose larvae develop within specific organs and tissues of their hosts. Infestations by these parasites are of significant veterinary importance due to their detrimental effects on animal health and productivity (Martín-Vega et al., 2025; Alahmed et al., 2002; Catts et al., 2002; Hilali et al., 2015; Ogbein et al., 2022; Hamed, 2022). In Kazakhstan, the most significant oestrid infestations affecting veterinary health are camel cephalopinosis, equine rhinoestrosis, and equine gasterophilosis. The prevalence of these infestations is linked to extensive pasture-based livestock management practices, resulting in substantial losses in health and productivity. Recent studies have

documented severe cases and outbreaks of equine gasterophilosis and camel cephalopinosis in western regions of Kazakhstan (Agronews.com, 2020; Caravan.kz, 2020; Inbusiness.kz, 2020; Tengrinews.kz, 2020; Pricom.kz, 2022).

Although these infestations have previously been reported in Kazakhstan (Omiralieva et al., 2017; Seitkamzina et al., 2023; Ybyrayev, 2009), current information on the species composition, geographic distribution, and seasonal dynamics of oestrid flies remains limited. Most previous studies have relied on morphological identification, while molecular data are scarce. Mitochondrial *cox1* sequencing can provide complementary evidence for species-level identification. The present study aimed to investigate the species composition, prevalence, and seasonal dynamics of oestrid infestations in camels and horses in the Atyrau and Kyzylorda Oblasts of Kazakhstan and to identify larval and adult specimens using mitochondrial *cox1* gene sequences.

Material and methods

Ethics statement

The objectives and procedures of this study were reviewed and approved by the Bioethics Committee of the Kazakh Scientific Research Veterinary Institute (20 October 2023). Field investigations, including the collection of bot fly larvae and the trapping of adult flies, were conducted with authorization from the Committee for Veterinary Control and Supervision of the Ministry of Agriculture of the Republic of Kazakhstan (Permit No. 17-03-05/2120, dated 13 August 2024). All procedures complied with the applicable legislation of the Republic of Kazakhstan and relevant guidelines on animal welfare, veterinary practice, and bioethics.

Study area and sample collection

Field investigations took place in June, July, and September of 2024 and 2025 in the Atyrau and Kyzylorda Oblasts of Kazakhstan, where recurrent cases of horse and camel mortality linked to oestrid larval infestations have been reported. A total of 169 animals were examined, including 91 camels for cephalopinosis, 68 horses for rhinoestrosis, and 10 horses for gasterophilosis. Animals were randomly selected from available herds and horse populations during field visits. Each animal was examined only once during the study period; no animals were re-examined in subsequent sampling periods. Therefore, all observations represented independent animals. The final sample size was determined by logistical and operational constraints associated with examining free-ranging animals maintained under year-round extensive grazing systems across the broad geographic area covered by the study. Camels and horses underwent endoscopic examination to detect oestrid larvae in the nasal cavities. In horses, this procedure was supplemented by oropharyngeal inspection and parasitological examination of the stomach. To complement the parasitological survey, adult oestrid flies were collected throughout the study period for morphological identification, molecular characterization, and assessment of the local oestrid fauna. Sixteen adult flies were collected in total.

All collected larvae were rinsed multiple times in distilled water to remove surface contaminants and subsequently preserved in 70% ethanol. Adult oestrid flies were also preserved in 70% ethanol and transported to the laboratory for further analysis (Grunin, 1955; Grunin, 1957; Grunin, 1953) under a SOPTOP SZX12 stereomicroscope.

Map preparation and GIS processing

The base layer of the map was generated using ArcGIS Pro (version 3.5.0, Esri, Redlands, CA, USA). The topographic map utilized built-in basemaps, and the country boundary shapefile was sourced from OpenStreetMap via Geofabrik (https://download.geofabrik.de/asia/kazakhstan.html). Open-source QGIS (https://qgis.org/) was used for preliminary data processing and verification.

Statistical analysis

All statistical analyses were performed in R (version 4.3.2). Infestation prevalence and corresponding 95% confidence intervals (CIs) were calculated for each stratified group using the Wilson score method, which provides more reliable interval estimates for small sample sizes and groups with zero prevalence. To evaluate the predictors of infestation probability while accounting for study design variations across sampling years, multivariable generalized linear modeling (GLM) with a binomial error distribution and logit link function was applied. To avoid

confounding and explicitly account for variations in study design across monitoring years, two distinct GLM specifications were implemented. First, an overall multi-year model (N = 159) was specified to evaluate global baseline effects across the full monitoring period:

$$\boldsymbol{logit(P) = \beta_0 + \beta_1(Species) + \beta_2(Region) + \beta_3(Year)}$$

where P represents the probability of host infestation. Seasonal terms were omitted from this overall formulation because structured intra-annual monitoring was restricted to 2025. Second, a dedicated seasonal dynamics model (N = 122) was constructed using exclusively the 2025 cohort:

$$\boldsymbol{logit(P) = \beta_0 + \beta_1(Period) + \beta_2(Species) + \beta_3(Region)}$$

The significance of individual predictors was assessed using Type II Wald x^2 tests. Post hoc pairwise comparisons were adjusted using Tukey's method to control the family-wise error rate. Effect sizes are presented as odds ratios (ORs) with 95% profile likelihood confidence intervals. When complete separation occurred because of zero-prevalence groups, Fisher's exact test was used. Statistical significance was set at $p < 0.05$.

Molecular genetic identification

Molecular identification of bot fly larvae and adult flies was based on PCR amplification of mitochondrial DNA markers followed by Sanger sequencing of the resulting amplicons.

DNA extraction

Genomic DNA was extracted using the standard phenol–chloroform protocol. Briefly, a small piece of tissue was excised from the mid-body region of each larval or adult specimen and thoroughly homogenized. The homogenate was incubated in extraction buffer containing Proteinase K at 56 °C for 2 h. DNA was then purified by phenol–chloroform extraction (25:24:1), precipitated with 70% ethanol, and resuspended in 1× TE buffer. The extracted DNA was stored at −20 °C until further molecular analyses.

PCR amplification

For the PCR, a conserved primer set targeting regions of the mitochondrial genome the cytochrome c oxidase subunit I (*cox1*) gene, was used. Six primer combinations were employed: UEA1/UEA8, UEA1/UEA10, UEA3/UEA8, UEA3/UEA10, UEA5/UEA8, and UEA5/UEA10. Mitochondrial amplicons of the expected size were successfully obtained from all isolates from bot flies (700 bp). The PCR reaction mixture consisted of HS-Taq PCR Biomasters (2×) (Biolabmix LLC, Novosibirsk, Russia), nuclease-free water, 10 pmol of each primer, and 20 ng of template genomic DNA. The amplification conditions for the DNA fragments are presented in Table 1. PCR amplification results were analyzed by 1% agarose gel electrophoresis using 1× TBE buffer supplemented with 8 μL of ethidium bromide.

Sequencing analysis and phylogeny

For subsequent sequencing, successfully amplified samples were selected. The obtained PCR products were purified using Exonuclease I (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Nucleotide sequence analysis was performed using the SeqStudio genetic analyzer (Thermo Fisher Scientific Applied Biosystems, USA). The resulting nucleotide sequences were manually inspected and edited using BioEdit software, followed by comparative analysis using the BLAST algorithm against reference sequences available in the GenBank database (https://www.ncbi.nlm.nih.gov/). Nucleotide sequences of isolates obtained in this study were deposited in GenBank, the numbers and GenBank accession numbers of all sequences are summarized in Table 2

Table 1: List of primers used in this study.

Target gene	Primer name	Primer sequence (5′ to 3′)	References
cox1	UEA1	F: GAATAATGAATAATTCCCATAAATAGATTTACA	Otranto et al. (2003), Zhang et al. (1997)
	UEA10	R: TCCAATGCACTAATCTGCCATATTA	
	UEA3	F: TATAGCATTCCCACGACGAATAAATAA	
	UEA5	F: AGTTTTAGCAGGAGCAATTACTAT	

Target gene	Primer name	Primer sequence (5′ to 3′)	References
	UEA8	R: AAAAATGTTGAGGGAAAAATGTTA	

Table 2: GenBank accession numbers of all sequences.

Species	New sequences, n	GenBank accession numbers generated in this study	Reference sequences used in the phylogenetic tree
Gasterophilus intestinalis	6	PZ448685, PZ448686, PZ448688, PZ448691, PZ448693, PZ448694	KR230406, KR230409, KR230415
Gasterophilus pecorum	3	PZ448689, PZ448690, PZ448692	MH553401, MH553402
Cephalopina titillator	1	PZ448687	MW167083, MZ209004
Rhinoestrus usbekistanicus	1	PZ449047	Not included because of insufficient reference sequences
Gasterophilus flavipes	0	-	MK412088, MK412089
Cuterebra grisea	0	-	KY928224, outgroup
Total	11	-	-

Phylogenetic analysis

Phylogenetic relationships were inferred from partial mitochondrial cytochrome *c* oxidase subunit I (*cox1*) sequences amplified with the UEA1/UEA10 primer pair. The sequences generated in this study were aligned with homologous *Gasterophilus pecorum*, *G. intestinalis*, *G. flavipes*, *Cephalopina titillator*, and *Cuterebra grisea* sequences retrieved from GenBank. Multiple sequence alignment was performed using the MUSCLE algorithm implemented in MEGA11 (Tamura and Nei, 1993; Tamura et al., 2021). A Maximum Likelihood (ML) phylogeny was reconstructed under the Tamura–Nei nucleotide substitution model. Rate heterogeneity among sites was modelled using a discrete Gamma distribution with five rate categories (+G; shape parameter = 2.1015). Node support was evaluated by bootstrap resampling, with bootstrap values indicated at the corresponding nodes. The *Cuterebra grisea* sequence (KY928224) was designated as the outgroup.

Results

Bot fly species recovered from camels and horses

Examination of 169 animals from the Atyrau and Kyzylorda oblasts revealed three species of oestrid bot fly larvae parasitizing camels and horses. In camels, second- and third-instar larvae (L2 and L3) of *C. titillator* were recovered from the nasal passages (Figures 1, and 2). In horses, third-instar larvae (L3) of *G. intestinalis* were collected from the stomach (Figure 3), whereas third-instar larvae (L3) of *G. pecorum* were recovered from the oropharynx (Figure 4). During the field surveys, 16 adult bot flies were collected and identified, comprising 15 *G. intestinalis* (nine females and six males) and one female *R.usbekistanicus* (5, and 6).

Prevalence of bot fly infestations

The prevalence of camel cephalopinosis during 2024-2025 is summarized in Table 3. Comparative analysis showed that the prevalence of *C. titillator* infestation remained relatively stable across the study regions throughout the study period, with an overall prevalence of 55.0% in 2024 and 52.1% in 2025.

To assess overall risk factors across the full dataset, the primary multi-year GLM (Model 1) was evaluated. Type II analysis of deviance using Wald's x^2 test identified host species as a significant predictor of infestation prevalence (likelihood-ratio $x^2 = 41.93$, $p < 0.0001$), whereas neither geographic region ($x^2 = 1.308$, $p = 0.2528$) nor year of observation ($x^2 = 0.490$, $p = 0.4840$) was significantly associated with infestation prevalence.

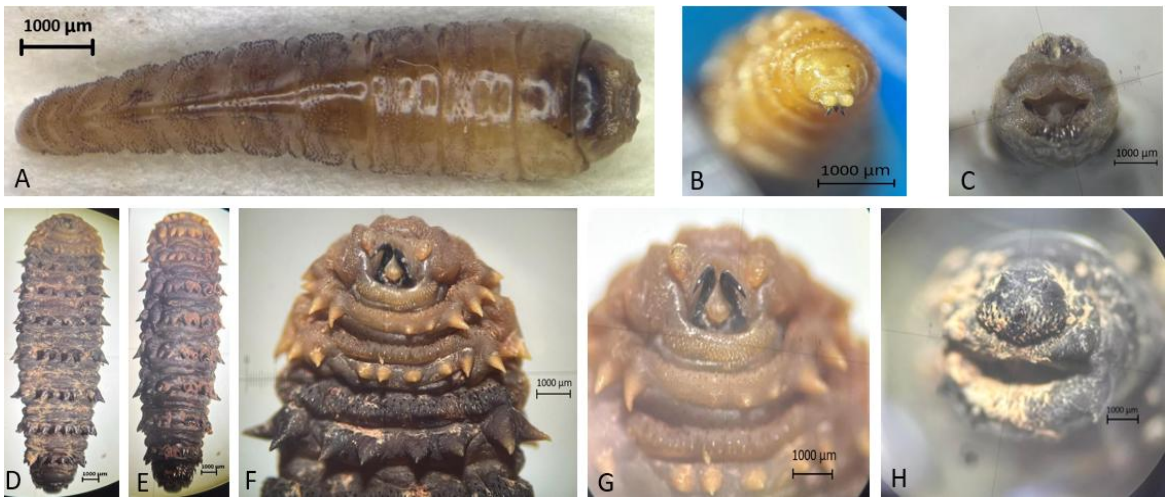

Figure 1: *C. titillator* larvae. A, C - second-instar larva (L2): (A) dorsal view, (B) pseudocephalon, (C) eighth abdominal segment, posterior view; D–H – third-instar larva: (D) dorsal view, (E) ventral view, (F) pseudocephalon, (G) mouth hooks, (H) eighth abdominal segment.

Figure 2: Endoscopic view of *C. titillator* larvae in the nasal passages of a camel.

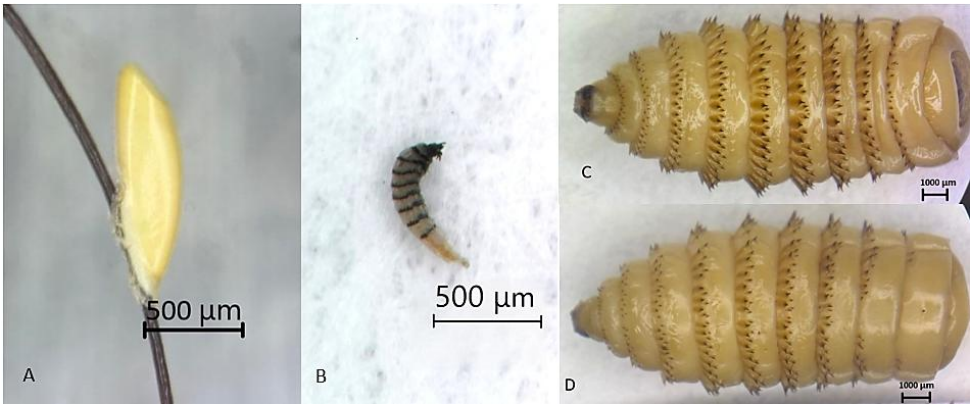

Figure 3: Developmental stages of *G. intestinalis*: (A) egg; (B) first-instar larva; (C) third-instar larva, ventral view; (D) third-instar larva, dorsal view.

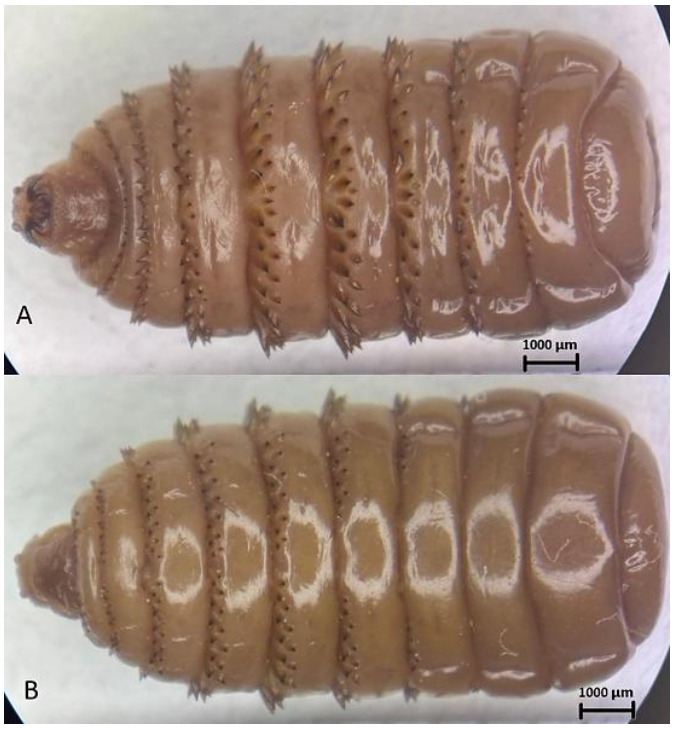

Figure 4: *G. pecorum* third-instar larva: (A) ventral view, (B) dorsal view.

Figure 5: Adult female of *G. intestinalis*.

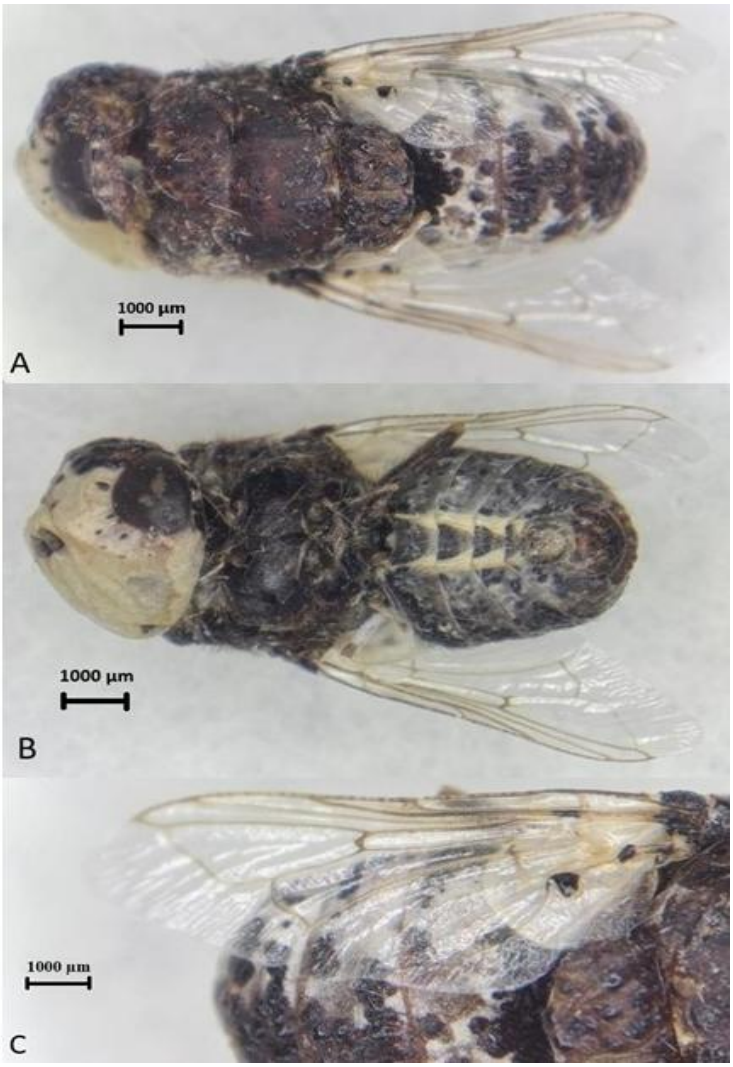

Figure 6: Adult female of *R. usbekistanicus*: (A) dorsal view, (B) ventral view, (C) wing.

In the Atyrau Oblast, the prevalence of camel cephalopinosis remained relatively stable, ranging from 54.5% to 56.6% over the study period. In contrast, a decline to 38.9% was observed in the Kyzylorda Oblast in 2025. The prevalence of rhinoestrosis in horses is presented in Table 4. Compared with camel cephalopinosis, rhinoestrosis occurred at a significantly lower prevalence, as confirmed by Tukey-adjusted post hoc pairwise comparisons (log OR = 2.71, SE = 0.517, z = 5.238, p < 0.0001). The adjusted odds ratio (OR) for horses relative to camels was 0.067 (95% CI: 0.022–0.169), indicating that the odds of detecting a bot fly infestation were 93.3% lower in horses than in camels. Overall prevalence decreased from 11.8% in 2024 to 5.8% in 2025. In addition, infestation intensity was consistently low in all infected horses, indicating the limited distribution of nasopharyngeal botflies in the studied equine populations. Analysis of regional variation showed that in the Atyrau Oblast, *C. titillator* infestation was detected in 36 of 64 examined camels, corresponding to a prevalence of 56.3% (95% Wilson CI: 44.3–67.5%). In the Kyzylorda Oblast, 12 of 27 camels were infested, yielding a prevalence of 44.4% (95% Wilson CI: 27.6–62.7%). However, geographic region was not a significant predictor of infestation prevalence in the GLM (log OR = 0.496, SE = 0.438, z = 1.132, p = 0.2576). Among horses, rhinoestrosis was recorded in 4 of 51 examined animals in the Atyrau Oblast (prevalence 7.8%; 95% Wilson CI: 3.1–18.5%) and in 1 of 17 animals in the Kyzylorda Oblast (prevalence 5.9%; 95% Wilson CI: 1.0–26.9%).

Seasonal dynamics of bot fly infestation

The results of the study on the seasonal dynamics of animal infestation are presented in Table 5. Multifactorial regression analysis using the seasonal GLM (Model 2) confirmed highly significant main effects for both sampling period ($\chi^2 = 17.37$, p < 0.001) and host species (χ^2 =

30.13, p < 0.001), while regional variations remained statistically non-significant (χ^2 = **0.329**, p = 0.566). The overarching regression model identified an explicit autumn decline in infestation probabilities across the board (OR = 0.134, 95% CI: 0.046-0.355), which reflects an 86.6% drop in the overall odds of detecting larvae by September compared to the early summer window.

Table 3: Prevalence of cephalopinosis in camels in 2024–2025.

Year	Oblast	Examined, n.	Infested, n.	Prevalence, %	Wilson CI, 95%
2024	Atyrau	11.0	6.0	54.5	[28.0, 78.7]
2024	Kyzylorda	9.0	5.0	55.6	[26.7, 81.1]
2024	Total	20.0	11.0	55.0	[34.2, 74.2]
2025	Atyrau	53.0	30.0	56.6	[43.3, 69.0]
2025	Kyzylorda	18.0	7.0	38,9	[20.3, 61.4]
2025	Total	71.0	37.0	52.1	[40.7, 63.3]

Table 4: Prevalence of rhinoestrosis in horses in 2024–2025.

Year	Oblast	Examined, n.	Infested, n.	Prevalence, %	Wilson CI, 95%
2024	Atyrau	14.0	2.0	14.3	[4.0, 39.9]
2024	Kyzylorda	3.0	0.0	0.0	[0.0, 56.1]
2024	Total	17.0	2.0	11.8	[3.3, 34.3]
2025	Atyrau	37.0	2.0	5.4	[1.5, 17.7]
2025	Kyzylorda	14.0	1.0	7.1	[1.3, 31.5]
2025	Total	51.0	3.0	5.8	[2.0, 15.9]

Table 5: Seasonal dynamics of bot fly infestations in animals in 2025.

Oblast	Animal species	Parasite	Study period	Examined, n	Infested, n	Prevalence, %	Wilson CI, 95%
Atyrau	Camels	*C. titillator*	June	33.0	25.0	75.8	[59.0, 87.2]
Atyrau	Camels	*C. titillator*	September	20.0	5.0	25.0	[11.2, 46.9]
Kyzylorda	Camels	*C. titillator*	July	8.0	6.0	75.0	[40.9, 92.9]
Kyzylorda	Camels	*C. titillator*	September	10.0	1.0	10.0	[1.8, 40.4]
Atyrau	Horses	*Rhinoestrus* sp.	June	16.0	0.0	0.0	[0.0, 19.4]
Atyrau	Horses	*Rhinoestrus* sp.	September	21.0	2.0	9.5	[2.7, 28.9]
Kyzylorda	Horses	*Rhinoestrus* sp.	July	5.0	1.0	20.0	[3.6, 62.4]
Kyzylorda	Horses	*Rhinoestrus* sp.	September	9.0	0.0	0.0	[0.0, 30.0]

The highest prevalence of *C. titillator* infestation in camels was recorded during summer. In the Atyrau Oblast, prevalence reached 75.8% in June, whereas in the Kyzylorda Oblast it peaked at 75.0% in July. By September, prevalence had declined to 25.0% and 10.0% in the Atyrau and Kyzylorda oblasts, respectively.

To account for zero-prevalence cells in horse cohorts, localized seasonal shifts were validated via Fisher's exact tests. For camels in Atyrau, the summer-to-autumn plunge was highly significant (Exact OR = 8.90, 95% CI: 2.23-42.36, p = 0.0005). Similarly, for camels in Kyzylorda, the drop was also highly significant (Exact OR = 20.67, 95% CI: 1.45-1357.58, p = 0.0128). A comparable seasonal pattern was observed for rhinoestrosis in horses, although infestation was limited to a few isolated cases and overall prevalence remained low. These findings indicate temporal variation in bot fly infestation prevalence across the sampling periods in the study regions of Kazakhstan, with prevalence being higher during the summer sampling periods than in September. The temporal dynamics of infestation and the corresponding odds ratios are illustrated in Figure 7.

Distribution patterns and seasonal activity of Gasterophilus spp. and Rhinoestrus spp.

Gasterophilosis was detected exclusively in horses from the Atyrau Oblast. In June and September 2025, 10 horses were examined using the partial helminthological necropsy method, and *G. intestinalis* larvae were detected in all examined animals.

Adult *G. intestinalis* activity and oviposition were observed during the first ten days of June and September 2025, corresponding to the periods of peak seasonal activity of stomach bot flies in the study area.

Adult *R. usbekistanicus* were collected in early September 2025 exclusively in the Kazaly district of the Kyzylorda Oblast. However, the larvae identified as belonging to the genus Rhinoestrus (Figure 8) could not be assigned to the species level based on morphological characteristics.

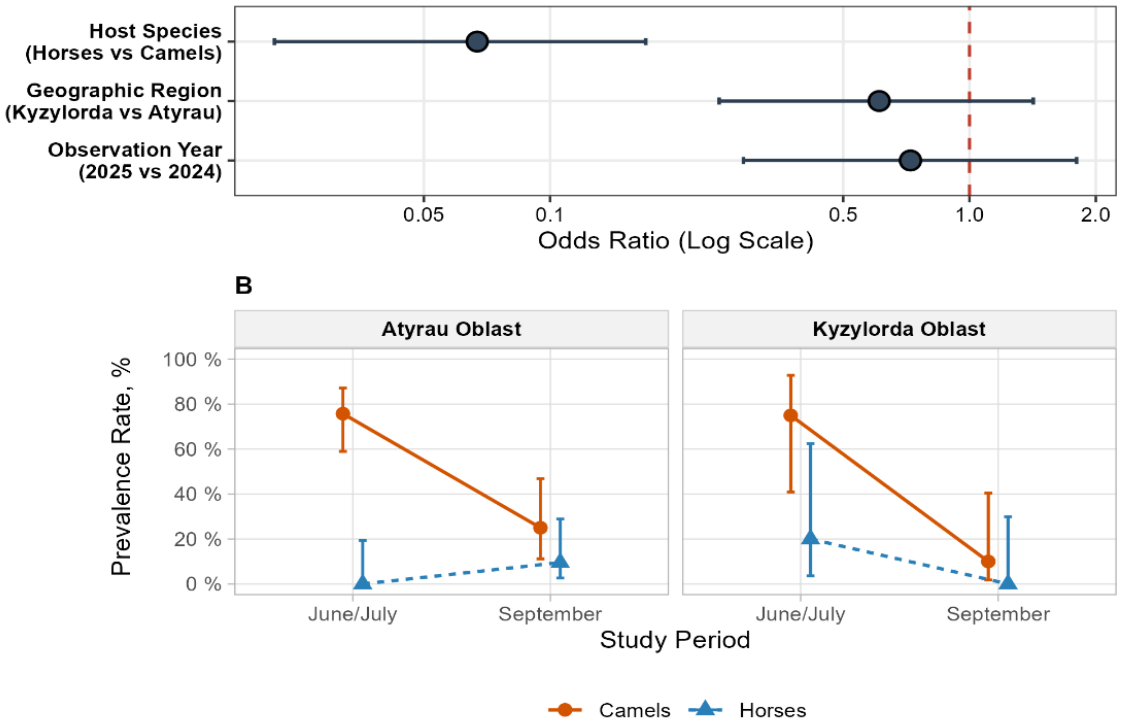

Figure 7: Prevalence odds ratios (A) and seasonal dynamics (B) of animal bot fly infestations with 95% confidence intervals.

Figure 8: Rhinoestrus sp. in nasal cavity of horses.

This limitation prevented a direct association between the collected adult flies and their larval stages, and therefore the occurrence of this species within the studied horse population could not be conclusively confirmed. Morphological and molecular identification of the larvae was not performed because their extraction from the ethmoid region during endoscopic examination was not feasible due to their deep localization in the posterior part of the nasal cavity.

Aberrant localization of Gasterophilus pecorum larvae

The present study revealed an unusual localization of *G. pecorum* larvae in horses from Atyrau Oblast. A similar phenomenon was previously reported in the same region in 1980–1981, and cases of this atypical parasitic manifestation have been recorded again in Atyrau Oblast since 2020, indicating its recurrence after more than four decades (Tengrinews.kz, 2020; Pricom.kz, 2022).

Unlike the typical localization of *G. pecorum* larvae within the gastrointestinal tract, numerous larvae were observed in the oral cavity and pharyngeal region of affected horses. Morphological examination confirmed that all collected specimens belonged to *G. pecorum*, and their species identity was further validated by molecular genetic analysis.

The aberrant localization was associated with severe clinical signs, including dyspnea, dysphagia, hypersalivation, impaired coordination, recurrent falls, and a forced dorsal recumbent position. In several cases, clinical signs of asphyxia were observed, resulting in fatal outcomes.

Molecular genetic identification and phylogenetic analysis of bot flies

Phylogenetic relationships were inferred from partial mitochondrial cox1 sequences amplified using the UEA1/UEA10 primer pair. Sequences generated in this study were aligned with homologous sequences of *Gasterophilus pecorum, G. intestinalis, G. flavipes, Cephalopina titillator*, and *Cuterebra grisea* retrieved from GenBank. Multiple sequence alignment was performed using the MUSCLE algorithm implemented in MEGA11. The Tamura–Nei nucleotide substitution model was selected because it accounts for unequal nucleotide frequencies and differences between transition and transversion rates, making it suitable for the analysis of mitochondrial cox1 sequences. Rate heterogeneity among sites was modelled using a discrete Gamma distribution with five rate categories (+G; shape parameter = 2.1015). The Maximum Likelihood tree was evaluated using 1,000 bootstrap replications, and bootstrap support values are shown at the corresponding nodes. The *C. grisea* sequence KY928224 was designated as the outgroup, confirming the correct rooting of the phylogenetic tree (Figure 9). The *G. intestinalis* sequences formed a well-supported clade together with the reference sequences KR230409, KR230415, and KR230406. The *G. pecorum* sequences grouped with reference sequences MH553402 and MH553401, confirming the species identity of the samples investigated. The *C. titillator* sequence obtained in the present study clustered with the reference sequences MZ209004 and MW167083, which also validates the accuracy of the morphological identification.

Molecular characterization of adult bot flies using the *cox1* marker amplified with primers UEA8 and UEA5 allowed identification of *R. usbekistanicus* and *G. intestinalis*. However, phylogenetic positioning of *R. usbekistanicus* was not performed due to the limited number of homologous sequences of this species available in the GenBank database.

Discussion

These findings demonstrate the persistence of several bot fly species in camel and horse populations in western Kazakhstan. The detection of *C. titillator*, *G. intestinalis*, and *G. pecorum* confirms that these species continue to constitute an important component of the parasitic fauna of domestic livestock in the region. Among the identified infestations, cephalopinosis was the most prevalent in camels. Rhinoestrosis in horses was characterized by a comparatively low prevalence. The observed seasonal patterns of infestation and adult fly activity are consistent with the biological characteristics of bot flies described for arid regions of Central Asia (Azizi et al., 2024; Zhang et al., 2023; Hilali et al., 2015).

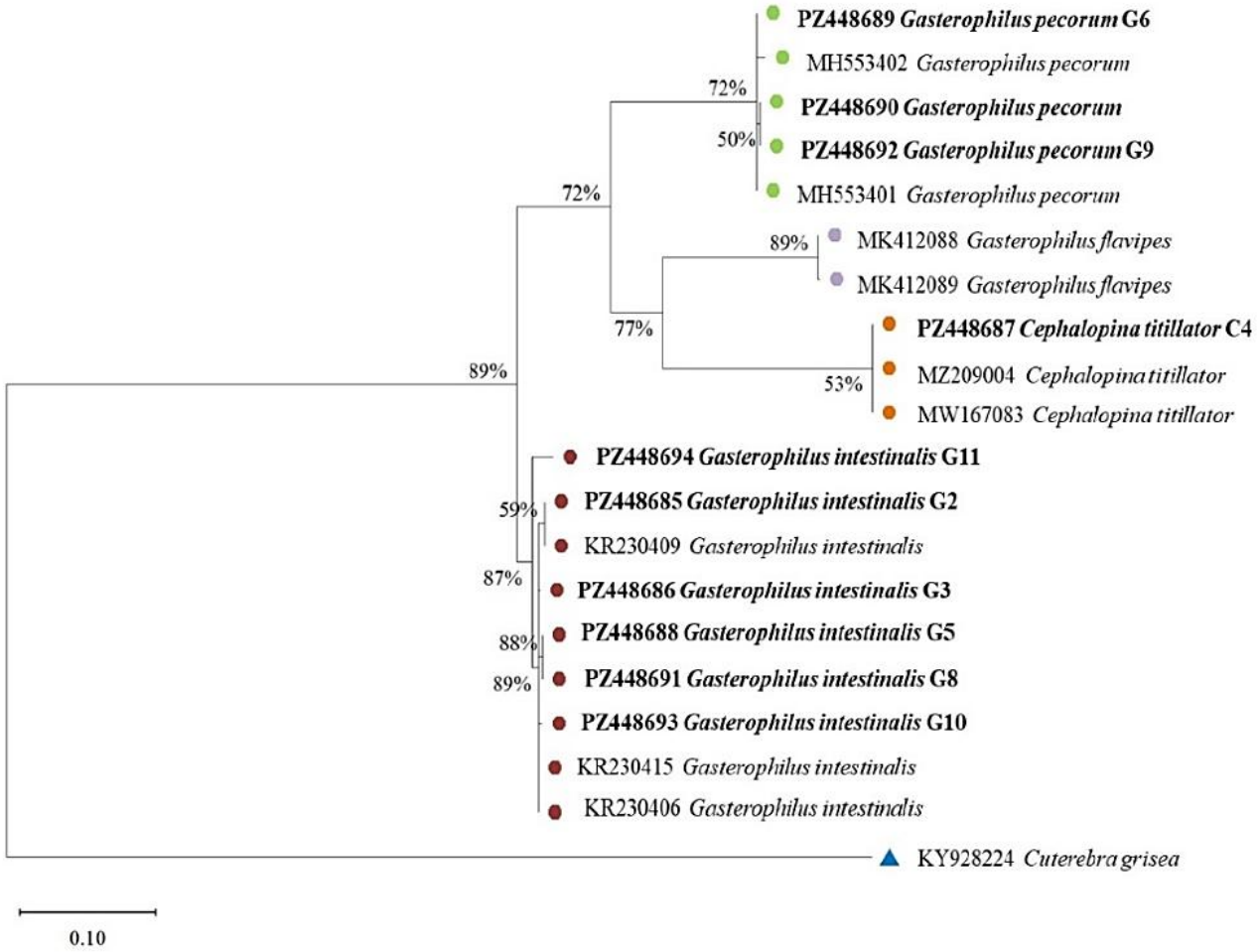

Figure 9: Maximum Likelihood phylogenetic tree of bot fly species based on partial mitochondrial *cox1* gene sequences (UEA1/UEA10). The scale bar represents 0.10 substitutions per nucleotide site. The sequence KY928224 *Cuterebra grisea* was used as the outgroup and is indicated by a blue triangle. Species are marked by different colours: green circles - *Gasterophilus pecorum*, dark red circles - *Gasterophilus intestinalis*, purple circles - *Gasterophilus flavipes*, and orange circles - *Cephalopina titillator*.

The detection of adult *R. usbekistanicus* in Kyzylorda Oblast is noteworthy, given the limited and fragmented information available on the distribution of this species in Central Asia (Abdel Rahman, 2022; Mahdy and Attia, 2021; Otranto et al., 2004). Our findings provide further evidence for the presence of adult *R. usbekistanicus* in western Kazakhstan and expand the current knowledge of the geographic distribution of this parasite.

An important finding of the present study is the recurrence of aberrant parasitism by *G. pecorum*, approximately 40 years after a similar phenomenon was described in the region by Rastegaev in 1990 (Rastegaev, 1990). During gasterophilosis outbreaks recorded in Atyrau Oblast in 2020–2021, numerous larvae were found in the oral cavity and pharynx of horses, representing an atypical localization that deviates from the normal developmental cycle of gastric bot flies.

This unusual localization was associated with pronounced clinical signs and high mortality among affected animals. The presence of numerous larvae may have caused mechanical obstruction and irritation of the upper respiratory and digestive tracts, resulting in impaired respiration and feed intake and ultimately contributing to the death of affected animals.

The recurrence of this phenomenon after four decades suggests that aberrant localization of *G. pecorum* larvae represents a periodically occurring yet epizootologically significant phenomenon in western Kazakhstan. Given its association with substantial livestock losses, further studies are required to elucidate the ecological and biological factors contributing to this atypical form of parasitism and to assess its impact on horse health and productivity.

Phylogenetic analysis based on mitochondrial

cox1 gene sequences confirmed the taxonomic assignment of the examined larvae and revealed distinct species-specific clustering patterns. The formation of separate clades for *G. intestinalis* and *G. pecorum* supports the utility of the 5′ region of the *cox1* gene as a DNA barcode for species-level identification within the genus Gasterophilus. Previous molecular studies have demonstrated that the *cox1* barcode region enables reliable discrimination among several Gasterophilus species, including *G. intestinalis*, *G. nasalis*, *G. nigricornis*, and *G. pecorum*. However, for closely related taxa, additional morphological and/or molecular approaches may be required for accurate species identification (Li et al., 2018).

In the reconstructed phylogenetic tree, *Gasterophilus intestinalis* sequences formed a well-supported clade together with reference sequences available in the GenBank database, confirming the reliability of the molecular identification. The relatively high bootstrap support values observed within this cluster indicate that the analyzed *cox1* fragment contains sufficient phylogenetic information for reliable discrimination of *G. intestinalis* from other species included in the analysis (Uakhit et al., 2025). Similarly, the clustering of the sequences obtained in this study with reference sequences of *G. pecorum* further supports their assignment to this species. The moderate bootstrap values observed for some internal nodes within the *G. pecorum* clade may reflect low levels of intraspecific genetic variation in the analyzed *cox1* region or the limited number of phylogenetically informative nucleotide substitutions (Otranto et al., 2005; Yao et al., 2025).

Although the partial mitochondrial *cox1* region provided sufficient resolution to support the morphological identification of the examined specimens, the phylogenetic results should be interpreted with caution. As a single, maternally inherited marker, *cox1* reflects the evolutionary history of one locus and may not fully represent the complete evolutionary history of the species. Furthermore, the relatively short sequence fragment and the limited availability of reference sequences - particularly for *R. usbekistanicus* and *C. titillator* - restrict the resolution of deeper relationships among oestrid lineages. Consequently, the close phylogenetic placement of *C. titillator* and Gasterophilus species should not be regarded as definitive evidence of a close higher-level evolutionary relationship. Additional analyses incorporating both mitochondrial and nuclear markers would be required to clarify deeper phylogenetic relationships among these taxa.

The observed clustering of *C. titillator* with representatives of the genus *Gasterophilus* may be explained by several factors. Both taxa belong to the family Oestridae and may retain similar conserved regions of the mitochondrial genome, particularly when relatively short fragments of the *cox1* gene are analyzed. Additionally, while a single mitochondrial marker offers high resolving power at the species level, it may be insufficient for fully resolving deeper phylogenetic relationships among closely related bot fly lineages. Finally, the number of available reference sequences for *C. titillator* in GenBank remains limited compared with the more extensively studied *Gasterophilus* species, which may also influence tree topology and bootstrap support values for individual nodes.

Taken together, these findings highlight the complexity of evolutionary relationships within the family Oestridae and demonstrate the importance of integrating morphological and molecular approaches for accurate parasite identification and epidemiological assessment. The detection of rarely reported species and the recurrence of aberrant larval localization emphasize the need for continued surveillance and comprehensive investigations into the ecological, climatic, and biological factors shaping bot fly distribution, transmission dynamics, and pathogenic effects in Central Asia

Conclusions

This study provides the first integrated morphological and molecular characterization of oestrid bot flies associated with horses and camels in Kazakhstan. Based on morphological examination and supported by *cox1* sequence data, four species were reliably identified at the species level: *C. titillator*, *R. usbekistanicus*, *G. intestinalis*, and *G. pecorum*. The concordant morphological and molecular evidence for adult *R. usbekistanicus* specimens further extends the known distribution of this species in Central Asia. A notable finding is the recurrence of aberrant larval localization of *G. pecorum* in the oral cavity and pharynx of horses, more than four decades after its previous report in western

Kazakhstan. The severe clinical manifestations and fatal outcomes associated with this condition underscore its substantial veterinary and epizootiological significance. Overall, these findings expand current knowledge of oestrid bot fly diversity and distribution in Kazakhstan and provide a foundation for continued surveillance, molecular epidemiological investigations, and the development of targeted control strategies.

Article Information

Funding. The data presented in this article were obtained with funding from the Ministry of Agriculture of the Republic of Kazakhstan BR22884615 «Scientific provision of epizootic welfare of fish and livestock farms of the Kepublic of Kazakhstan on infectious and parasitic diseases».

Conflicts of Interest. The authors declare no conflict of interest.

Authors contribution. All authors contributed equally to work.

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