BioControl (2024) 69:387–395
https://doi.org/10.1007/s10526-024-10267-0

Predatory behavior and potentiality of *Toxorhynchites* spp. (Diptera: Culicidae) against *Aedes* mosquito

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Received: 5 October 2023 / Accepted: 19 May 2024 / Published online: 31 May 2024

Abstract *Toxorhynchites* mosquitoes (Diptera: Culicidae), also known as elephant mosquitoes or mosquito eaters, are the largest mosquitoes globally and do not bite humans but prey on other mosquito larvae. We used a dipping and pipetting methods to collect larvae of *Toxorhynchites* spp. and *Aedes* mosquitoes from discarded tires. In a laboratory study, we investigated how container type, water volume, and depth affect the predation behavior of *Toxorhynchites* spp., and we also examined its predation efficiency during both day and night. Our research revealed that an individual *Toxorhynchites* spp. larva can consume up to 45 *Aedes* larvae, with mean predation of 25.18 (±9.35) larvae in 24 h. Interestingly, predation rates and impacts remained consistent regardless of container type, water volume, or depth. While predation was more active during the day, there were no significant differences between daytime and nighttime predation abilities. These findings highlight *Toxorhynchites* spp. larvae as a promising biological control agent for mosquito management in various settings, independent of seasonal or light conditions, especially for the *Aedes* spp.

Keywords *Aedes* · Biological control · Predation · *Toxorhynchites* · Vector management

Handling Editor: Marta Montserrat.

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Introduction

Mosquitoes, belonging to the order Diptera and family Culicidae, are common insects found worldwide, except in Antarctica (Hawkes and Hopkins 2022). As of January 2024, 113 genera and 3726 species of mosquitoes have been recorded globally (Harbach 2024). Fortunately, less than 10% of mosquito species spread human diseases (Yee 2022), and among them, the three genera (*Anopheles*, *Aedes* and *Culex*) comprise significant mosquito vectors of different diseases (Tandina et al. 2018). *Anopheles* mosquitoes are vectors for malaria (Sinden 2007; Ross 2024), a potentially fatal disease caused by *Plasmodium. Culex* mosquitoes are vectors for lymphatic filariasis (Manson-bahr 1962; Haynes 2001), a chronic inflammatory disease caused by filarial worms. *Aedes* mosquitoes transmit more than 26 arboviruses and filarial worms to various animals, including humans (Kraemer et al. 2015; Tippelt et al. 2020). Climate, vegetation, and human activities are key determinants of the distribution of these mosquitoes (Sukupayo et al. 2024). The genus *Aedes* comprises around 970 species (Harbach 2024), including the medically important *Aedes aegypti* (Linnaeus, 1762) and *Aedes albopictus* (Skuse, 1895). These two *Aedes* mosquito species are effective carriers of lethal disease-causing agents such as the chikungunya virus, dengue virus, yellow fever virus, zika virus, and others, resulting in a considerable negative impact on human health and significant economic losses worldwide (Mordecai et al. 2020). Among different arboviral diseases transmitted by *Aedes* mosquitoes, dengue fever is the most significant mosquito-borne viral disease worldwide. This group of mosquitoes caused millions of disease cases with thousands of deaths in 2022 (ECDC 2023). *Aedes aegypti* and *Aedes albopictus* mosquitoes are the principle vectors responsible for transmitting dengue disease in numerous urban areas across Southeast Asia (Smith 1956; Mohamad and Zuharah 2014). These vectors are known to breed in containers, whether natural or artificial, found in and around human settlements (Naish et al. 2014; Zuharah and Sumayyah 2019). *A. aegypti* is native to the African continent, whereas *A. albopictus* is native to the Southeast Asian forest (Russell et al. 1969; Tedjou et al. 2020). *A. aegypti* mainly breeds indoors and outdoors, while *A. albopictus* typically breeds outdoors, although it is well adapted to the peridomestic environment (Ebi and Nealon 2016). Both species are well adapted to human habitation for oviposition and larval development in natural and artificial water bodies (Naish et al. 2014). Although both feed from morning until dusk, *A. albopictus* also bites at night, indicating its role in transmitting diseases throughout the day and night (Higa 2011). Since dengue vaccines are still being developed, the most effective method currently available to prevent this disease remains controlling the mosquito vector (Mohamad and Zuharah 2014). Various pesticides have been used for mosquito control. However, this approach presents challenges such as rising environmental pollution, chemical and labor costs and public reluctance to have chemicals in their domestic water containers (Focks 2007). Additionally, mosquito vectors have resisted several pesticides (WHO 2012). Consequently, the rising threat of *Aedes*-borne diseases necessitates urgently exploring alternative vector control methods specifically targeting these mosquito species.

In this context, the use of biological control agents, such as mosquitoes from the genus *Toxorhynchites* (Diptera), is one of the most promising alternatives to chemical methods in controlling container-breeding mosquitoes like *Aedes*. Within the diverse realm of mosquito species, a unique paradox emerges in the form of *Toxorhynchites*. These mosquitoes are the largest in the world, often referred to as "elephant mosquitoes" or "mosquito eaters" because of their size and long trunk-like proboscis (Focks 2007). The genus *Toxorhynchites* comprises approximately 100 species distributed across four subgenera (Harbach 2024) and demonstrates an impressive global distribution (Tyagi et al. 2015). They are found across various continents, including in countries such as Indonesia, India, and Thailand in Asia, and Tanzania in Africa. In North America, these mosquitoes can be found in locations such as Florida, USA and Mexico (Tyagi et al. 2015). Notably, species like *Toxorhynchites splendens* exhibit an even wider distribution, extending to countries like Bangladesh, Nepal, Myanmar, and Sri Lanka (Tyagi et al. 2015).

Adult *Toxorhynchites* mosquitoes do not feed on blood. Instead, they only feed on nectar from flowers or fruits using their sizeable and downwardly curved proboscis (Collins and Blackwell 2000; Focks 2007), making them harmless to humans. Unlike other blood-hungry females, *Toxorhynchites* mosquitoes pack a protein punch from their larval stage, allowing them to lay eggs without needing a single blood meal (Watts and Smith 1978; Mercer et al. 2005).

Interestingly, the larvae are dynamic predators, preying on various aquatic invertebrates, including mosquito larvae (Padgett and Focks 1981; Focks 2007; Millado and Sumalde 2018), making them natural regulators of mosquito populations. The *Toxorhynchites* spp. larvae are ambush predators, meaning that they wait quietly for prey to come to them (Zuharah et al. 2015). These voracious hunters do not actively search, but once the prey comes close, they unleash their powerful mandibles in a swift strike (Zuharah et al. 2015; Vinogradov et al. 2022). The entire process happens quickly, with the captured prey consumed within a few minutes (Steffan and

Evenhuis 1981; Zuharah et al. 2015). This ambush-hunting behavior makes *Toxorhynchites* larvae natural regulator of mosquito populations, consequently helping to control the spread of numerous mosquito-borne diseases (Focks 2007; Schiller et al. 2019; Vinogradov et al. 2022).

Toxorhynchites mosquitoes show a distinct preference for laying their eggs in a wide variety of both artificial and natural containers, similar to their prey, *Aedes aegypti* and *A. albopictus* (Trpis 1972; Nyamah et al. 2011). Moreover, almost any container holding water can serve as a breeding site, especially if it is partly shaded (Trpis 1972; Donald et al. 2020). Turbid water in both immature stages and predator–prey interactions efficiently attracts female *T. splendens* for egg deposition (Phasomkusolsil et al. 2022). Surprisingly, bacterial by-products of *Toxorhynchites* feeding attract *Aedes* mosquitoes, drawing them to containers containing *Toxorhynchites* larvae (Albeny-Simões et al. 2014). These factors highlight the significance of *Toxorhynchites* mosquitoes in controlling *Aedes* larvae. However, there is a lack of clarity regarding how the type of container, water depth, and water volume affect the reproductive behavior of *Toxorhynchites*. Therefore, this research aims to investigate how container type, water depth, volume, and even diurnal variations impact the predatory behavior of *Toxorhynchites* against *Aedes* larvae. Understanding these influences is crucial for optimizing the utilization of naturally occurring *Toxorhynchites* spp. as biological control agents. The experiment could potentially lead to widen our understanding on *Toxorhynchites* spp. and their predatory behavior in different environmental settings. Such information is needed to control mosquitoes as well as reductions in mosquito-borne diseases, particularly in low and middle-income countries such as Nepal, where cost-effective measures are essential for disease prevention.

Materials and methods

Collection and rearing of larvae

In July 2023, predatory larvae of *Toxorhynchites* spp. and prey larvae of *Aedes* spp. were collected using a dipping and pipetting methods from discarded tires near Tamakoshi Bazar (27° 37′ 2.82″ N, 86° 04′ 37.23″ E, 875 m asl) along Lamosangu-Ramechhap Highway in Dolakha, Nepal. In addition to the larvae collected in Dolakha, more *Aedes* larvae were collected from Sipadol (27° 39′ 8.01″ N, 85° 26′ 33.00″ E, 1380 m asl), Bhaktapur. All collected larvae were kept in separate plastic bottles and brought to the Nepal Academy of Science and Technology laboratory, Lalitpur, Nepal. In the laboratory, the collected fourth-stage larvae of *Toxorhynchites* spp. were placed in a glass container (beaker) and left for 24 h to acclimatize them to the new laboratory conditions. *Aedes* larvae were also introduced into the containers as their food source.

On the other hand, the collected third and second-stage larvae of *Toxorhynchites* spp. were reared in a single beaker to facilitate their transformation into fourth-stage larvae. *Aedes* larvae served as their primary food source during this period. After the emergence of fourth-stage larvae, the experiment was repeated for each larva as mentioned above.

Impact of container types, water volumes, and depth on *Toxorhynchites* spp. predation

We investigated how different container types and variations in water volumes and depths could influence predation behavior within controlled laboratory conditions. This is because the *Aedes* spp. are container-breeders and the current data of predatory behavior of *Toxorhynchites* spp. on the former species in new environment can be translated for prevention strategies. The laboratory environment was maintained at a temperature of 25 ± 2 °C. Three different types of containers were selected: glass tumblers (14.5 cm height × 6.5 cm diameter), glass bowls (7 cm height × 16 cm diameter), and plastic bottles (13.5 cm height × 9.5 cm diameter). Each container type was filled with specific volumes of dechlorinated water, resulting in varying water depths. For the glass tumblers, 100 ml, 200 ml, and 300 ml with corresponding water depths of 4.0 cm, 8.0 cm, and 12.0 cm, respectively, were used. The glass bowls were filled with 150 ml and 300 ml of water. The water depths were 1.0 cm and 2.0 cm, respectively. Similarly, the plastic bottles were filled with 150 ml and 300 ml of water, with depths of 1.5 cm and 3.0 cm, respectively. Thus, the experiment included three container types, four different water volumes (100 ml, 150 ml, 200 ml, and 300 ml), and seven different water depths (1.0 cm, 1.5 cm, 2.0 cm, 3.0 cm, 4.0 cm, 8.0 cm, and 12.0 cm). The acclimatized fourth-stage *Toxorhynchites* larvae were individually introduced to each container alongside 50 *Aedes* larvae as prey to initiate the experiment formally. After 24 h, we observed and recorded the number of prey consumed by the predator. We carefully noted two key points: First, the predation rate, which refers to the number of prey killed and consumed by *Toxorhynchites* spp. within a specific time period (e.g., per day). It reflects the rate of actual prey consumption by the predator. Second, the predatory impact, which is the total number of prey killed by *Toxorhynchites* spp. within a specific time period, regardless of whether they were consumed or not. The predatory impact captures the broader effect of the predator on the prey population, including potential "compulsive killing" behavior observed before pupation. We conducted four replicates of the experiment. The experiment included a single control treatment without a predator. This control group utilized a glass tumbler filled with 200 ml of water at a depth of 8 cm. Both the control and experimental groups were given 10 mg of finely powdered fish food to feed the prey larvae.

Impact of day and night time on *Toxorhynchites* spp. predation efficiency

To evaluate the predation efficiency of *Toxorhynchites* spp. during both daytime and nighttime, eight experimental setups, described in the previous section were prepared and replicated over three consecutive days and nights. The study focused on distinguishing diurnal and nocturnal predation behaviors, with data collection occurring from 6 am to 6 pm representing daytime observations and from 6 pm to 6 am, representing nighttime observations. The glass windows were left uncovered during both day and night to allow natural light exposure, and no artificial light was provided at night to maintain a realistic nocturnal environment. Including eight replicates ensures a robust statistical foundation for assessing predation efficiency during these distinct temporal periods.

Statistical analysis

The effects of container type, water depth, and water volume on the predation rate and predatory impact of *Toxorhynchites* spp. preying on *Aedes* spp. were analyzed separately using generalized linear models (GLM) with Poisson distribution of errors and a log link function. Additionally, Mann–Whitney U test was employed to assess day-night differences in predation rates and predatory impacts. All analyses were performed with SPSS version 20.0, and figures were created using GraphPad Prism Version 5.00.

Results

Predation rate and predatory impact

The fourth instar larvae of *Toxorhynchites* spp. were each fed 50 *Aedes* spp. larvae, and their consumption was recorded over 24 h for four days. A total of 40 observations were collected. The descriptive analysis of the observed data yielded a mean predation rate ($\pm$SE) of an individual *Toxorhynchites* spp. larva of 23.29 ± 1.73. Similarly, the mean predatory impact within 24 h of a single *Toxorhynchites* spp. was 25.18 ± 1.77. Furthermore, no mortality was observed in the control group, and *Aedes* mosquitoes proceeded to complete development into adulthood in the lack of the predatory *Toxorhynchites* spp.

Effect of container types on the predation rate and predatory impact of *Toxorhynchites* spp.

In the experiment, a total of 28 containers were used, including 12 glass tumblers, eight glass bowls, and eight plastic bottles. Predation rate and predatory impact were calculated for 24 h. The mean predation rate of *Toxorhynchites* spp. on *Aedes* species was highest (26.25 ± 3.10) in glass bowls compared to other containers, but difference was not statistically significant ($\chi^2 = 2.47$, df = 2, $p = 0.29$) (Fig. 1a). A similar result was found for predation impact (28.13 ± 3.18) in glass bowls, which was also not statistically significant ($\chi^2 = 2.25$, df = 2, $p = 0.33$) (Fig. 1b).

Effect of water depth on the predation rate and predatory impact of *Toxorhynchites* spp.

In the experiment seven different water depths, ranging from 1 to 12 cm, were used to investigate the impact on the predation rate and predatory impact of *Toxorhynchites* larvae on *Aedes* larvae within 24 h. The mean predation rate was the highest

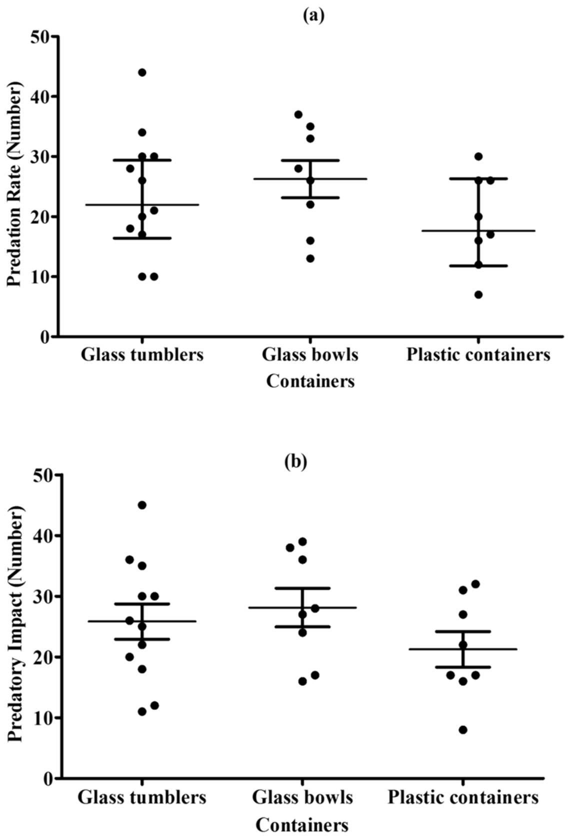

Fig. 1 Predation rates (mean ± SE) **(a)** and predatory impact (mean ± SE) **(b)** of *Toxorhynchites* spp. preying on *Aedes* spp. in different container types (dots indicate observed data)

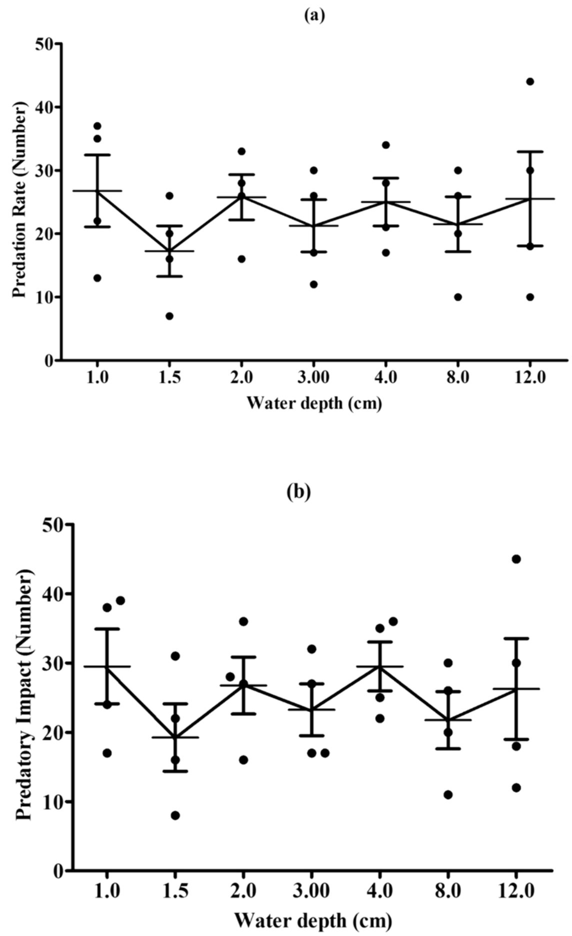

Fig. 2 Predation rates (mean ± SE) **(a)** and predatory impacts (mean ± SE) **(b)** of *Toxorhynchites* spp. on *Aedes* spp. in different water depths (dots indicate observed data)

(26.75 ± 5.66) in a water depth of 1 cm compared to others without any statistical significance ($\chi^2 = 2.94$, df = 6, p = 0.82) (Fig. 2a). In addition, the mean predatory impact was highest and equal at water depths of 1 cm (29.50 ± 5.39) and 4 cm (29.50 ± 3.52). However, data were insignificant ($\chi^2 = 3.77$, df = 6, p = 0.71) (Fig. 2b).

Impact of water volumes on the predation rate and predatory impact of *Toxorhynchites* spp.

In this study, four water volumes, ranging from 100 to 300 ml, were used to assess the impacts of *Toxorhynchites* larvae predation on *Aedes* larvae within 24 h. The mean predation rate was highest in the 100 ml water volume (25 ± 3.76) compared to other three volumes. Nevertheless, there was no statistical significance ($\chi^2 = 0.45$, df = 3, p = 0.93) (Fig. 3a). Similarly, the mean predatory impact was also highest (29.50 ± 3.52) in the 100 ml water volume compared to others, without any statistical significance ($\chi^2 = 1.53$, df = 3, p = 0.68) (Fig. 3b).

Predation efficiency at daytime and nighttime

Among the eight setups that ran continuously for three days and nights, the mean predation rate and predatory impact were assessed for those periods. The mean predation rate during the daytime was slightly higher than at night (12.38 ± 1.18 *vs*. 10.13 ± 0.94) without any statistical significance (p = 0.20) (Fig. 4a). Similarly, the mean predatory impact during the day was slightly higher than at night (13.13 ± 1.24 *vs*. 10.63 ± 0.90) without any significant difference (p = 0.15) (Fig. 4b).

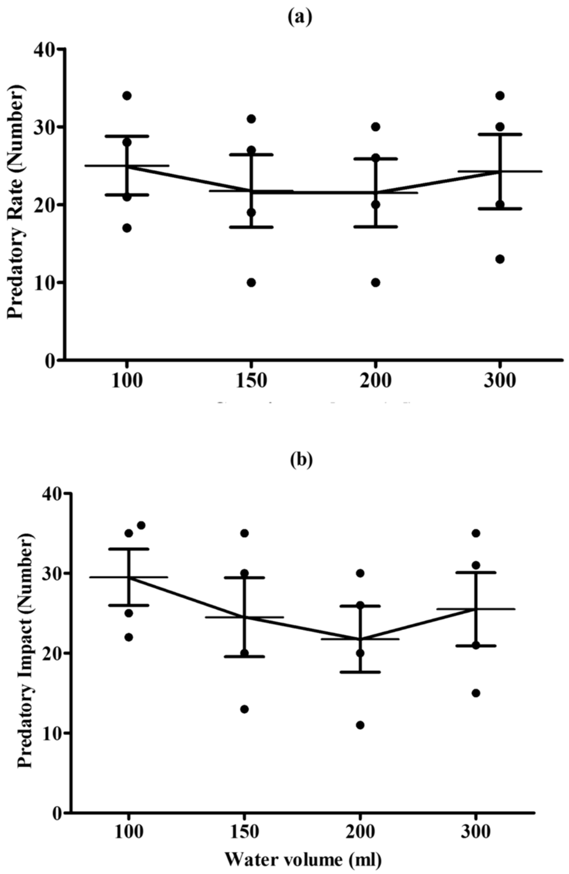

Fig. 3 Predation rates (mean ± SE) **(a)** and predatory impact (mean ± SE) **(b)** of *Toxorhynchites* spp. on *Aedes* larvae in different water volumes (dots indicate observed data)

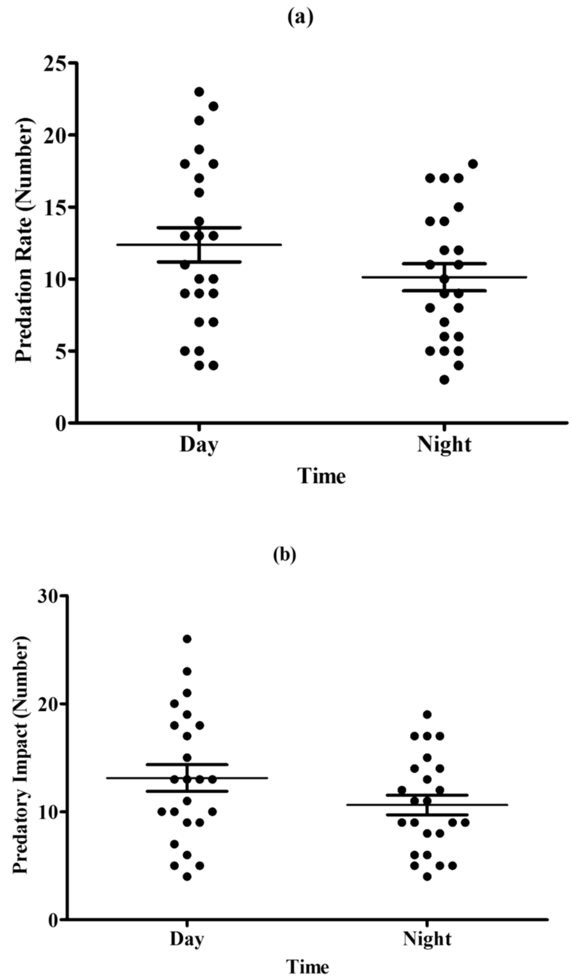

Fig. 4 Predation rates (mean ± SE) **(a)** and predatory impact (mean ± SE) **(b)** for day and night treatments (dots indicate observed data)

Discussion

The occurrence of *Toxorhynchites splendens* in Nepal has been previously documented based on morphological characteristics (Peters and Dewar 1956; Darsie et al. 1996). Consistent with these earlier finding, our morphological examination suggested the current specimens as *T. splendens*. However, we have designated them as *Toxorhynchites* spp. because polymerase chain reaction and sequencing analysis are still currently underway.

The feeding strategy *Toxorhynchites* spp. play a significant role in efficiently managing prey populations. Their feeding behavior is commonly described as "opportunistic" because these predatory larvae do not actively hunt for prey. Instead, they lay in wait and ambush potential prey that meets their mandibles (Zuharah et al. 2015). Research suggests that a single *Toxorhynchites* larva can consume a significant number of other mosquitoes, even hundreds, while it is growing (Mohamad and Zuharah 2014; Digma et al. 2019; Vinogradov et al. 2022). *Toxorhynchites* species like *T. amboinensis*, *T. rutilus septentrionalis*, and *T. splendens* have a behavior called "prepupal compulsive killing" (Steffan and Evenhuis 1981; Russo 1986; Vinogradov et al. 2022). This behavior involves their larvae getting rid of potential prey before it turns into pupae, even though they do not eat it.

Our study found a mean predation of 25.18 (±9.35) *Aedes* larvae consumed by a single

Toxorhynchites larva within 24 h. The highest number of prey consumed by a single larva in our experiment was 45. This exceeds the previously reported mean predation of 17.68 with a maximum consumption of 23 larvae per 24 h (Digma et al. 2019). However, the average predation observed in our experiment is lower than the mean predation of 51.67 (±2.52) with a maximum consumption of 54 larvae reported in a study from India (Malla et al. 2023).

The type of container was previously thought to impact predation (Padgett and Focks 1981; Mohamad and Zuharah 2014), but our study found no significant differences in container types. *Toxorhynchites* larvae are opportunistic predators. When they encounter mosquito larvae in a given container, they are likely to feed on them. This opportunistic behavior means that the container type is less important than the presence of prey.

While some studies have suggested that the volume of the search area affects the mean number of prey larvae consumed by *T. splendens* larvae (Malla et al. 2023), our findings contradict this, as we have observed that the volume of water does not have a significant effect on predation. This is consistent with earlier research findings (Dominic and Das 1998; Mohamad and Zuharah 2014). These results indicate that *Toxorhynchites* spp. can effectively control *Aedes* larvae regardless of water volume, making them suitable for dry and rainy seasons.

Additionally, *Toxorhynchites* spp. displayed consistent diurnal and nocturnal predation potentialities, aligning with previous findings from Banjarbaru, Indonesia (Muhamat et al. 2022). This implies that their efficiency in regulating prey populations is not influenced by environmental light intensity, making them suitable for daytime and nighttime control. While no mortality was observed in the control treatment, a limitation of this study is the absence of control groups for each combination of water container type, depth, and volume. A full factorial design with separate control groups for each condition would have strengthened the isolation of the predator's specific effects on prey mortality.

When considering the use of *Toxorhynchites* spp. as a biocontrol agent for *Aedes* species, it is important to recognize some limitations. These challenges include limitation of natural container breeding sites, difficulties in captive rearing, and the time-consuming nature of evaluating predation efficiency in natural habitats (Donald et al. 2020). Additionally, there is a risk of disease spread associated with *Toxorhynchites* spp., as they sometimes stockpile their prey (Frank et al. 1984). This highlights the need for detailed studies to confirm their predation characteristics and ensure their effectiveness as a control method.

The ongoing fight against *Aedes*-borne diseases demands innovative and sustainable control strategies. Our study demonstrated that *Toxorhynchites* spp. are effective biological control agents for *Aedes* mosquitoes. Their predation behavior remained consistent across different container types, water depths, and volumes. This adaptability suggests they can thrive in various aquatic environments throughout both dry and rainy seasons. Furthermore, consistent predation under both daylight and nighttime conditions suggests minimal impact from environmental factors like light, making them effective predators throughout the day and night. This approach offers a valuable alternative considering the lack of effective vaccines for *Aedes*-borne diseases like dengue, chikungunya, and zika.

Acknowledgements We express our gratitude to Nirmal Bajracharya for assisting in the statistical analysis. We extend our appreciation to the Central Department of Zoology, Tribhuvan University, Bhaktapur Multiple Campus, Tribhuvan University, and the Nepal Academy of Science and Technology for providing research opportunity. We acknowledge the local government authorities and residents of the study areas for their consistent support during the field survey.

Authors contributions Punya Ram Sukupayo (conceptualization-lead, data curation-lead, formal analysis-lead, investigation-lead, methodology-lead, validation-lead, visualization-lead, writing–original draft-lead, writing–review and editing-lead), Ram Chandra Poudel (conceptualization-supporting, supervision-supporting, validation-supporting, writing–original draft-supporting, writing–review and editing-supporting), Tirth Raj Ghimire (conceptualization-supporting, supervision-supporting, writing–original draft-supporting, writing–review and editing-supporting). All authors read and finalized the submission.

Funding This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Declarations

Conflict of interest The authors have no financial or proprietary interests in any material discussed in this article.

Research involving human and animal rights The study did not involve human participants and/or vertebrate animals.

Informed consent The study contains no individual person's data in any form. Informed consent is not applicable.

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Punya Ram Sukupayo This research is part of a PhD project of Punya Ram Sukupayo. He is working on *Aedes* spp. and other mosquitoes and their biocontrol mechanisms.

Ram Chandra Poudel is involved in molecular characterization of endemic, threatened, and economically important flora and fauna. He is investigating plant and animal diseases and their vectors via molecular tools.

Tirth Raj Ghimire is working on One Health Approach of Understanding Diseases. He is investigating how pathogens and vectors are distributed in an environment, and how they modify hosts' immune system, and how natural products and drugs/vaccines work against these pathogens.