Journal homepage:https://www.environcj.in/

Environment Conservation Journal

ISSN 0972-3099 (Print) 2278-5124 (Online)

Preliminary investigation of mud-puddling behavior in *Appias* spp. (Albatross butterfly) in the Aralam Wildlife Sanctuary, India

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ARTICLE INFO

Received : 18 October 2023
Revised : 31 December 2023
Accepted : 04 January 2024

Available online: 01 March 2024

Key Words:
Albatross butterflies
Aralam Wildlife Sanctuary
Butterfly mud-puddling
Mud-puddling aggregation
Soil nutrient compositions
Visual cues

ABSTRACT

Butterflies represent one of the most popular and readily identifiable insect taxa. The annual migration of *Appias* spp. (Albatross butterflies) is common in the Aralam Wildlife Sanctuary (AWS), India. During migration, the butterflies moving along the stream course often settle down in masses across a mud-puddling site. This study sheds light on the multifaceted interplay between soil parameters and the mud-puddling aggregation behavior observed in Albatross butterflies within the AWS. The results revealed no significant differences in most soil nutrients between the mud-puddling sites (MS) and control sites (CS). However, the moisture content and available sodium content were significantly greater in the MS treatment ($p = 0.0003$ and $p = 0.002$, respectively). Hence, more moisture (avg_35.95% in MS than avg_0.99% in CS) and more sodium (avg_34 mg/kg in MS than avg_15.11 mg/kg in CS) were found to play significant roles in the behavior of certain species, such as *A. albina*, *A. wardii*, and *A. lyncida*. The majority of the tested soil parameters exhibited weak correlations with the density of butterflies (the r values of pH, moisture, N, C, S, Cu, Cd, Zn and Mn were -0.48, -0.59, -0.35, -0.30, -0.36, +0.16, -0.18, -0.18 and -0.005, respectively). Notably, sodium and potassium were the sole minerals exhibiting a strong positive correlation with the density of mud-puddling *Appias* spp. ($r = +0.78$ and $r = +0.77$, respectively), emphasizing the significance of sodium and potassium in shaping the density of Albatrosses engaged in mud-puddling behavior. Many butterflies employ visual cues to locate these resources. Hence, this study also explored the preference of *Appias* spp. for conspecific paper decoys for directing butterflies to puddle sites. The presence of conspecific decoys noticeably drew butterflies, underscoring the significance of visual cues in butterfly behavior. Additional studies in this domain could enhance our understanding of butterfly ecology and the mechanisms governing their decision-making processes.

Introduction

Mud puddling is a behavior commonly observed in numerous adult lepidopteran species, particularly males, where they gather on various damp surfaces, such as moist ground, perspiration, tears, excrement, and decaying animal remains (Boggs and Dau, 2004). This behavior serves as a means for butterflies to acquire essential nutrients, particularly sodium and protein (Beck *et al*., 1999). This process is similar to 'salt licking' performed by higher terrestrial vertebrates (Bhatade *et al*., 2019). The scarcity of sodium in the diets of many Lepidopterans species is believed to have driven the

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Doi:https://doi.org/10.36953/ECJ.25752732

evolution of mud puddling, as it provides an opportunity to obtain this vital mineral (Boggs and Dau, 2004). Notably, the benefits of mud puddling extend beyond individual nutritional needs. Mud puddling has connections with both the nutritional ecology and reproductive biology of insects (Smedley, 2009). Adult male butterflies accumulate sodium during mud puddling, which is then transferred to their mates through spermatophores during copulation (Molleman, *et al*., 2005; John and Tennent, 2012). This exchange of sodium has potential effects on female egg production and offspring fitness, representing a nuptial gift from the male to the female and highlighting the broader significance of mudpuddling behavior (Molleman, *et al*., 2005; Lewis and South 2012). In various flying insect species, adult males frequently form aggregations (Boggs and Jackson, 1991), often referred to as "mud puddling clouds," primarily comprising young males (Watanabe, 2016). These aggregations, known as leks, can involve hundreds of individuals and are believed to enhance mating success for males within the aggregations (DuVal *et al*., 2018). However, reports in the literature also document instances of females engaging in individual mud puddling (John and Dennis, 2019) or, in some cases, only females being found at sites with perspiration or wet sand (Boggs and Jackson, 1991). The choice of puddling resources at these sites is influenced by factors such as the species' mating system, competitive abilities, the current nutrient status of individuals, and environmental conditions (Beck *et al*., 1999). Hence, sites with suitable salt concentrations for puddling become scarce and unevenly distributed. Butterflies employ visual and olfactory cues, depending on their family, to locate these resources (Beck *et al*., 1999).

Among butterfly species, mud-puddling aggregations of *Appias* spp. (Albatross butterflies) have been frequently observed in the hills of southern India (Mathew and Binoy, 2002). They are highly migratory and fly at altitudes ranging from 900 m to 1200 m (Sreekumar and Balakrishnan, 2001). These butterflies exhibit notable behavior of visiting moist areas in significant numbers, and when they gather near streams in forested areas, they form sizeable male aggregations on surfaces consisting of sand, silt, and mud (Sreekumar and Balakrishnan, 2001). During migration, swarms of common Albatross butterflies are often accompanied by individuals from other species, including the painted sawtooth, great orange tip, common blue bottle, common jay, and blue momon (Palot *et al*., 2015). These mixed swarms can comprise 2-20 members and typically fly at heights of 2-6 feet above the ground (Palot *et al*., 2015). As they migrate along stream courses, these butterflies frequently settle in significant numbers at mudpuddling sites (Palot *et al*., 2015). Notably, the Aralam Wildlife Sanctuary (AWS) in Kerala experiences annual occurrences of large mud-puddling aggregations of Albatross butterflies (Palot *et al*., 2015). Despite the regular use of annual butterfly surveys in the AWS during the migratory season by the Forest Department and local nongovernmental organizations, the reasons behind the formation of these substantial mud-puddling aggregations of *Appias* spp. have not been thoroughly investigated, especially with regard to soil. Thus, there is a gap in our understanding of the ecological factors driving the occurrence of such aggregations. In this context, the present study sought to explore the soil parameters and nutrients that facilitate the formation of mud-puddling aggregates in *Appias* spp. within the limits of the AWS. By addressing this specific topic, our aim is to advance our understanding of the factors that influence mud-puddling behavior and to provide insights into the ecological dynamics and significance of mud-puddling aggregations in this particular butterfly species.

Material and Methods

Study Area

The Aralam Wildlife Sanctuary (AWS), located between 11°50′ and 11°52′ N and 75°57′ and 75°59′ E, is the most northern protected area in Kerala, spanning an area of 55 square kilometers (Fig. 1). This sanctuary is situated in the Aralam and Kottiyoor villages within the Kannur District. It shares borders with the Brahmagiri Wildlife Sanctuary in Karnataka State to the north, the reserve forests of Wayanad to the east, and the Central State Farm of Aralam to the west (Palot *et al*., 2015). One notable feature of AWS is the significant aggregation of migratory butterflies that gather in

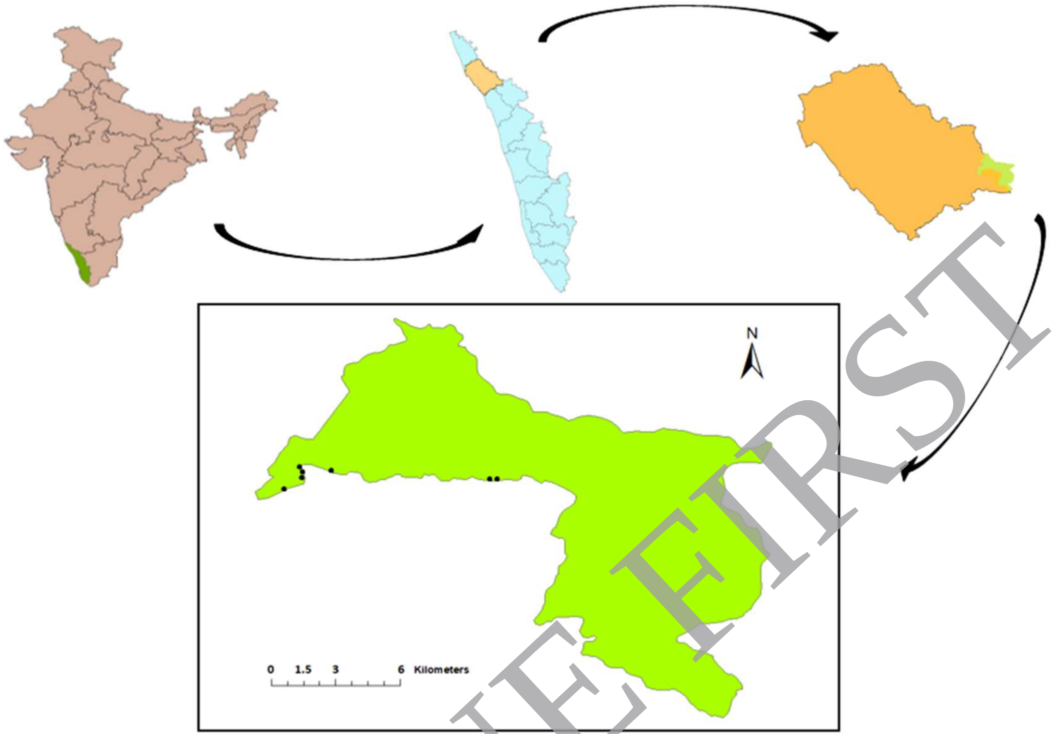

Figure 1. Map of Aralam WLS, Kerala, India (study sites are highlighted with black dots)

riparian areas and riverbeds during the summer months (Palot *et al*., 2015). The main mud-puddling leks occur near the riverbeds of Cheenganniipuzha and Uruttipuzha (Palot *et al*., 2015). The arrival of the common Albatross butterfly (*Appias albina*) in December marked the beginning of migratory leks, which continued until March or April in the study area (Palot *et al*., 2015).

Butterfly observation and soil sampling

The methodology employed for this study involved the purposive random sampling method of study quadrats in the identified mud-puddling sites (MS), with an equal number of control quadrats placed in control sites (CS). The study quadrats where butterfly mud-puddling behavior was observed. The control quadrats were positioned 5 meters away from the puddling sites, perpendicular to the flowing river where no puddling was observed. The established quadrats remained permanent throughout the data collection period. Observations focused on three species of albatross butterflies: *A. albina* (Common Albatross), *A. wardii* (Indian Albatross), and *A. lyncida* (Chocolate Albatross) (Fig. 2). The following seven MSs and equal numbers of CSs were identified: Valayamchal (MS 1, CS1), Pookundu (MS 2, CS2, MS3, CS3, MS4 and CS4), Narikadavu (MS 5, CS5), and Kariankappu (MS 6, CS6, MS7 and CS7). A random 3 m × 3 m quadrat was set up at each site and marked with sticks in the early morning to avoid disturbing the butterflies once their congregation began. The peak activity hours for Albatross migration, between 11:00 am and 01:00 pm, were chosen for monitoring. The number of Albatross species within each MS was recorded. The butterfly observations were carried out only from the MS since no butterfly mudpuddling behavior was observed in the CS. The counts were cross-checked with on-site photographs. Replicate data were collected for five sampling days at each site. The collected data were analyzed using Microsoft Excel, and the density, frequency, and abundance of butterflies were calculated using equations 1, 2, and 3, respectively.

Figure 2. Mudpuddling aggregation by *Appias albina* at the study site

$$\text{Density} = \frac{\text{Total number of Individuals}}{\text{Total number of quadrats X quadrat area}} \quad (1)$$

$$\text{Abundance} = \frac{\text{Total number of individuals}}{\text{Number of quadrat in which species occured}} \quad (2)$$

$$\text{Frequency} = \frac{\text{Number of quadrat in which species occured}}{\text{Total number of quadrat}} X\, 100 \quad (3)$$

Soil sampling involved collecting samples of mud, muddy water, or soaked soil from the uppermost 5 cm of soil at each MS and its corresponding CS (5 meter away from the puddling sites, perpendicular to the flowing river). These samples were immediately placed in Ziplock bags to retain moisture and were subsequently submitted to a soil testing center within 24 hours. The soil analysis was conducted by the Environmental Monitoring Service in Auroville and the Central Instrumentation Facility at Pondicherry University. Various parameters, such as pH; moisture content; and sodium, potassium, nitrogen, carbon, sulfur, copper, zinc, cadmium, and manganese concentrations, were analyzed. Pearson's correlation analysis between soil parameters and butterfly density were done to determine the contribution of respective soil parameters in shaping butterfly mud-puddling aggregates in MS. Standard T test was applied to determine the significant difference in the soil parameters between MS and CS.

Decoy experiments:

A decoy experiment was carried out at three sites within the study area: Valayanchal, Pookkundu, and Narikkadavu. The experimental setup consisted of two quadrats measuring 2 m × 2 m each, positioned 1.5 meters apart from each other on the streambank. Observations were conducted between 10:00 am and 2:00 pm, which is the period of peak butterfly activity, under sunny conditions with temperatures ranging from 30-40°C. The observations were made at a distance of 2 meter from the quadrats. Only individuals who entered the test quadrats were counted. The purpose of the experiment was to investigate the impact of visual cues on the puddling behavior of the common albatross butterfly and its association with other species. A decoy of the common bluebottle, a widespread mud-puddling swallowtail species, was utilized to test this association. In the first treatment, 8-10 artificial decoys of common albatross, whose wings were in a semiclosed position, were placed in one of the two quadrats at each test site. The same methodology was applied for the second treatment, except that the positions of the decoys were switched to the alternate quadrat, eliminating any location preferences (Otis *et al*., 2006). In the third treatment, 3-5 common Albatross decoys were placed in one test quadrat, while 3-5 common Bluebottle decoys were placed in the other quadrat, providing freely flying butterflies with a choice between the two decoy species. Therefore, no empty control puddles were used in the third treatment. To avoid double counting of the same butterfly individual, a separate observer was assigned to conduct parallel observations. A statistical test (chi-square) was performed to evaluate the affinity of the butterflies for artificial decoys.

Results and Discussion

Associations between soil parameters and mud-puddling behavior

Most of the soil nutrients did not significantly differ between MS and CS, as indicated by the p values obtained (Table 1). The p values for pH, K, N, C, S, Cu, Cd, Zn, and Mn were 0.31, 0.13, 0.08, 0.12, 0.05, 0.17, 0.14, 0.41 and 0.48, respectively, and all of them were above the significance threshold, suggesting that these nutrients were similar in both MS and CS (Table 1). However, the t test results revealed significant differences in moisture content ($p = 0.0003$) and sodium content ($p = 0.002$) between the two types of sites (Table 1). Hence, more moisture (avg_35.95% in MS than avg_0.99% in CS) and more sodium (avg_34 mg/kg in MS than avg_15.11 mg/kg in CS) were found to play significant roles in the behavior of certain species, such as *A. albina*, *A. wardii*, and *A. lyncida*, regardless of the other soil nutrient compositions. The average values of pH, K, N, C, S, Cu, Cd, Zn and Mn were 7.53, 28 mg/kg, 0.33%, 3.87%, 0.08%, 0.04%, 0.005%, 0.06% and 1.04%, respectively, in MS and 7.43, 35.17 mg/kg, 0.43%, 2.57%, 0.11%, 0.01%, 0.003%, 0.05% and 0.99%, respectively, in CS. The Albatross butterfly densities obtained in MS1, MS2, MS3, MS4, MS5, MS6 and MS7 were 1.53n/m², 4.46n/m², 2.07n/m², 5.26n/m², 4n/m², 4.27n/m² and 3.2n/m², respectively (Table 2). Most of the soil parameters did not show a strong correlation with butterfly density (Table 3). The r values for pH, moisture content, nitrogen, carbon, sulfur, copper, cadmium, zinc, and manganese were -0.48, -0.59, -0.35, -0.30, -0.36,+ 0.16, -0.18, -0.18 and -0.005, respectively (Table 3). The concentrations of sodium and potassium strongly positively correlated with the density of *Appias* spp. butterflies ($r = +0.78$ and $r = +0.77$, respectively) at the study site (Fig. 1, Table 3). The linear regression models exhibited a goodness of fit of 60% for sodium and 59% for potassium, further emphasizing the significance of sodium and potassium in shaping the density of Albatrosses engaged in mud-puddling behavior (Fig. 1).

Table 1: p value between mud-puddling sites and control sites (highlighted in bold indicates significance)

Test parameters	p value
pH_MS - pH_CS	0.31
Moist_MS - Moist_CS	**0.0003**
Na_MS - Na_CS	**0.002**
K_MS - K_MS	0.13
N_MS - N_CS	0.08
C_MS - C_CS	0.12
S_MS - S_CS	0.05
Cu_MS - Cu_CS	0.17
Cd_MS - Cd_CS	0.14
Zn_MS - Zn_CS	0.41
Mn_MS - Mn_CS	0.48

These findings suggest that a high moisture content combined with a high level of sodium could be contributing factors to the puddling behavior of butterflies near streams or rivers. Interestingly, the study showed that Albatrosses species never aggregated away from streams or river banks, even when there was not much variation in soil nutrients between different sites. This observation suggested that sites closer to rivers with more moisture were found to play a significant role in the behavior of certain species, such as Albatrosses, regardless of the soil nutrient composition. The moisture content in the soil is the key factor for mud-puddling butterflies (Bhatade *et al*., 2019). It is generally observed that butterflies are attracted to muddy areas with higher moisture contents, as these areas could provide a suitable environment for puddling activities. The main function of moisture content is to dissolve soil nutrients so that butterflies can obtain them in aqueous form (Bhatade *et al*., 2019).

Table 2: Results of butterfly sampling

Butterfly sampling	MS1	MS2	MS3	MS4	MS5	MS6	MS7
Total number of butterflies	23	70	31	79	60	64	48
Density (n/m²)	1.53	4.67	2.07	5.26	4	4.27	3.2
Abundance (n)	5.75	17.5	6.2	26.33	12	12.8	12
Frequency (%)	80	80	100	60	100	100	80

Table 3: Pearson's correlation between soil parameters and butterfly density (highlighted in bold indicates a strong correlation)

Soil parameters	Pearson's r
pH	-0.48
Moisture content	-0.59
Available sodium	**+0.78**
Available potassium	**+0.77**
Available nitrogen	-0.35
Available carbon	-0.30
Available sulphur	-0.36
Available copper	+0.16
Available cadmium	-0.18
Available zinc	-0.18
Available manganese	-0.005

Hence, the significant difference in the proportion of moisture content between the study sites and control sites may have influenced the availability of nutrients in dissolved form for uptake by butterflies. In the UK, *Pieris* spp. butterflies also exhibit mud-puddling aggregation behavior similar to that of *Appias* spp. (Dennis *et al*., 2014). Previous studies have suggested that mud-puddling behavior involves the intake of various minerals, including sodium, calcium, phosphorus, and protein (Beck *et al*., 1999; Boggs and Dau, 2004). For example, Lai-Fook (1991) reported that calcium and phosphorus are taken in during mud-puddling, and Beck *et al.* (1999) reported the intake of protein. Likewise, a large body of literature describes sodium utilization by various mud-puddling butterflies (Beck *et al*., 1999; Boggs and Dau, 2004; John and Tennent, 2012). A study by Boggs and Dau (2004) revealed soluble sodium concentration as the driving factor for mud-puddling behavior in *P. napi,* which is similar to the findings of other studies suggesting that sodium is the major factor attracting butterfly mud-puddling aggregation in butterflies (Smedley and Eisner, 1995, 1996). The present study confirmed earlier findings that sodium plays a significant role in the mud puddling of butterflies.

According to a study by Inoue *et al.* (2015), swallowtail butterfly males also use mud puddling to eliminate too much potassium acquired from the host plant leaves. Butterflies are also known to excrete an equivalent amount of potassium while acquiring sodium from the soil (Smedley and Eisner, 1996). This could also explain not only the sodium but also the significant amount of potassium found in the soil, where a high density of *Appias* spp. butterfly mud puddling occurs. This could explain why, in addition to primary triggering of the mineral sodium, potassium was correlated with butterfly density at the study sites. An increase in the concentration of sodium and potassium at puddling sites corresponds to an increase in butterfly density. To further investigate the relationships of sodium and potassium with butterfly density, linear models were employed (the results for sodium are $Y = 0.1264 - 0.7443x$, where Y represents butterfly density and x represents the quantity of sodium; the results for potassium are $Y = 0.104 + 0.651x$, where Y represents butterfly density and x represents the quantity of potassium in puddling sites) (Fig. 3). This equation can be used to predict butterfly density at mudpuddling sites in Aralam if the concentrations of sodium and potassium are known.

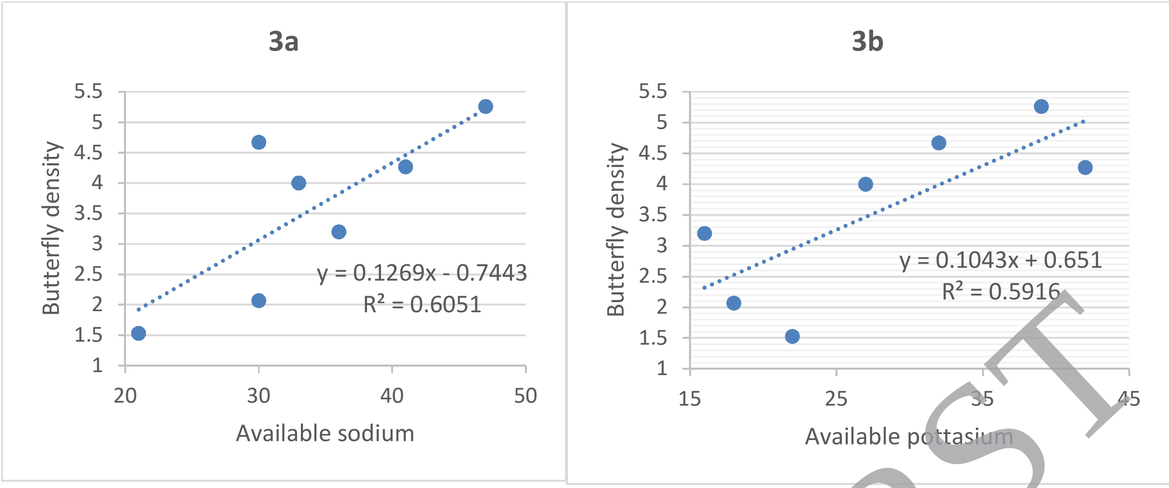

Figure 3. Scatter plot showing linear regression of butterfly density against sodium (3a) and potassium (3b) concentrations

Decoy Experiment

The present study provides valuable insights into the preference of the common albatross using visual cues to find potential puddling sites. Chi-square (χ^2) analysis showed strong evidence of common Albatross affinity toward artificial decoys (p = 0.003) (Table 4). The significant difference observed in the statistical analysis underscores the importance of conspecific decoys in attracting common albatrosses (Fig. 4). The preference for conspecific decoys over interspecific decoys suggests that visual cues, such as the presence of conspecifics, play a crucial role in butterfly behavior (Otis *et al.*, 2006). Furthermore, we noted potential spatial variation in the affinity for paper decoys. The greater number of butterflies attracted to the conspecific decoy in the second treatment (the treatment in which the decoy position was switched to the alternate quadrat) than in the first treatment indicates that the effects of conspecific decoys on butterfly behavior may vary depending on the specific location. In addition to visual cues, the presence of olfactory cues (sodium) or humidity (moisture content) is also important for attracting butterflies. This spatial variation is an important aspect to consider when investigating butterfly behavior and resource utilization.

Table 4. Chi-square test results showing the affinity of *Appias albina* for decoys (highlighted in bold indicates significance)

Decoy type	Only Albatross butterflies decoy in either of the quadats	Albatross butterflies decoy in one and Bluebottle butterflies in the other quadrat
Chi-square	16.29	0.67
p value	**0.003**	0.414

In addition to the common Albatross, other butterfly species belonging to the families Pieridae and Papilionidae showed an affinity for paper decoys. Although this aspect was not included in the data analysis, it aligns with previous research that has reported the use of visual cues, such as the number and presence of brightly colored conspecifics, in locating puddle sites among Papilionoids and Pierids (Beck *et al.*, 1999). This finding suggested that the

Figure 4. Albatross individuals (red arrows) puddling near to Albatross artificial decoys (green arrows)

utilization of visual cues for resource acquisition is a common behavior across different butterfly species. This understudied aspect of butterfly ecology holds significant potential for expanding our understanding of butterfly behavior and the mechanisms underlying decision-making processes. While the present study provides a preliminary investigation of this phenomenon, future research efforts should further explore this phenomenon to unravel the specific mechanisms and implications of spatial and visual bias in butterfly behavior. Overall, these findings enhance our understanding of butterfly ecology and behavior by emphasizing the importance of considering multiple factors, such as soil characteristics and visual cues, in the study of mud-puddling behavior. Continued investigations in this field will deepen our knowledge of butterfly behavior and contribute to effective conservation efforts.

Conclusion

In conclusion, this study provides valuable insights into the factors that influence mud-puddling behavior in butterflies. The analysis of soil nutrients revealed that, with the exception of moisture content and sodium levels, most parameters did not significantly differ between mud-puddling sites and control sites. The higher moisture content and high sodium levels of mud-puddling sites suggest their potential role in attracting butterflies near streams or rivers. Correlation analysis indicated a positive relationship between sodium and potassium concentrations and butterfly density at puddling sites, emphasizing the major role of sodium and potassium in mudpuddling behavior compared to that of other minerals investigated at the AWS. Notably, the common Albatross individuals exhibited a strong preference for conspecific decoys, highlighting the significance of visual cues in their resource utilization. Overall, these findings enhance our understanding of butterfly ecology and behavior by emphasizing the importance of considering multiple factors, such as soil characteristics and visual cues, in the study of mud-puddling behavior. Continued investigations in this field will deepen our knowledge of butterfly behavior and contribute to effective conservation efforts

Acknowledgments

The author (JA) is thankful to Kishor Hari for assisting us throughout the field survey. The authors express our gratitude to the Forest Department, Govt. of Kerala, for granting permission and providing assistance throughout the field survey at AWS.

Conflict of interest

The authors declare that they have no conflicts of interest.

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