



Reproductive behaviour of the Kloof Frog (*Natalobatrachus bonebergi*), an endangered South African endemic

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ABSTRACT

Amphibians are vulnerable to extremes in environmental factors such as air temperature, rainfall and relative humidity which greatly influence their physiological parameters and subsequently their phenology. This vulnerability extends to reproductive behaviour and is reflected in developmental processes of larvae, which are laid in jelly-like eggs. *Natalobatrachus bonebergi* is an Evolutionarily Distinct and Globally Endangered (EDGE) species and is also monotypic within the genus. Its threat status is based on the threats faced by this species and a severely fragmented distribution. This species is a riparian forest specialist - threatened by deforestation, urbanisation, agricultural clearing, pollution and siltation. Little knowledge exists on the reproductive behaviour of *N. bonebergi*. Monitoring and surveillance efforts for the species have been undertaken since 2013 in Vernon Crookes Nature Reserve, KwaZulu-Natal, South Africa. Using a protocol developed by the Endangered Wildlife Trust's Threatened Amphibian Programme and tested by Ezemvelo KZN Wildlife Honorary Officers, extensive data have been collected on *N. bonebergi* reproduction in the form of egg clump counts along riverine transects. With this wealth of data, this study aimed to collect additional data on *N. bonebergi* reproduction through acoustic monitoring, egg clump isolation experiments and *in situ* observations of reproductive behaviour from April 2023 to April 2024. A quantitative approach was used to statistically investigate various reproductive behaviours and the relationships these have with environmental factors throughout the species' extended breeding season. Numerous new insights into parental care, male competition, complex communication and interactions between conspecifics and responses to environmental stimuli have been gathered. Knowledge of this species' phenology is important in understanding the evolutionary and ecological processes governing their behavioural patterns, aiding conservation management of this species and its habitats.

Keywords

amphibian, behaviour, bioacoustics, conservation, monitoring, reproduction

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TABLE OF CONTENTS

i	ABSTRACT	2
ii	ACKNOWLEDGEMENTS	3
iii	LIST OF FIGURES.....	6
iv	LIST OF TABLES	10
1	INTRODUCTION	14
1.1	Global amphibians in decline	14
1.2	South African amphibians in decline	17
1.3	Monitoring in amphibian conservation.....	18
1.4	Bioacoustics and acoustic monitoring	20
1.5	Challenges of amphibian conservation in South Africa	23
1.6	<i>Natalobatrachus bonebergi</i> : conservation, life-history information and study objectives	24
2	MATERIALS & METHODS	28
2.1	Study area.....	28
2.2	Egg clump monitoring	28
2.3	Egg clump isolation experiments	30
2.4	Acoustic monitoring	31

2.5	Observations of behaviour	32
3	RESULTS	34
3.1	Egg clump monitoring	34
3.1.1	Egg clump monitoring	34
3.1.2	Egg clump isolation experiments	54
3.2	Acoustic monitoring	67
3.2.1	Call profiles.....	67
3.2.2	Passive acoustic monitoring	79
3.3	Observations of behaviour	86
4	DISCUSSION	99
4.1	Egg clump monitoring	99
4.1.1	Egg clump monitoring protocol	99
4.1.2	Egg clump isolation experiments	101
4.2	Acoustic monitoring	102
4.2.1	Call profiles.....	102
4.2.2	Passive acoustic monitoring	103
4.3	Observations of behaviour	104
4.3.1	Visual behaviours	104
4.3.2	Multimodal behaviours.....	106
4.4	Conclusions.....	107

5	BIBLIOGRAPHY	109
6	ANNEXURE: Short communication article published in the African Journal of Ecology	116

LIST OF FIGURES

Figure 1. Map of Vernon Crookes Nature Reserve, KZN, indicating the egg clump monitoring transect (white dots connected with a line) and the SM4 PAM site (white star)	28
Figure 2. Egg clump isolation cage constructed around newly laid egg clump with wire and plastic mesh. iButton hygrometers were used to measure temperature (°C) and relative humidity (%)	30
Figure 3. Female <i>N. bonebergi</i> attempting to reach egg clump. Swollen abdomen indicates that this female absorbed water to transport to egg clump	30
Figure 4. SM4 Song Meter by Wildlife Acoustics	31
Figure 5. Bar graph of total egg clumps per attachment surface for the 2013–2017 timeframe	34
Figure 6. Bar graph of total egg clumps per attachment surface for the 2018–2023 timeframe.	35
Figure 7. Bar graph of total egg clumps binned in height categories of 10 cm intervals for the entire dataset (2013–2023).....	36
Figure 8. Line plot of mean egg clump height for each month of the 2013–2017 timeframe .	37
Figure 9. Line plot of mean egg clump height for each month of the 2018–2023 timeframe .	37
Figure 10. Bar graph of the highest recorded egg clumps each year from 2013 to 2023	38
Figure 11. Dual-axis bar graphs depicting monthly rainfall overlayed with the number of eggs recorded in the 2013–2017 and 2018–2023 timeframes respectively. Number of eggs are depicted as black bars and monthly rainfall is depicted as a red line.....	39

Figure 12. Boxplot of monthly rainfall (mm) vs the stage of development over the entire dataset (2013- 2023)	40
Figure 13. Scatterplot of monthly rainfall (mm) regressed with height of egg clumps over the entire dataset (2013–2023).....	40
Figure 14. Scatterplot of monthly rainfall (mm) regressed over the number of egg clumps recorded over the entire dataset (2013–2023)	41
Figure 15. Scatterplot of monthly rainfall (mm) regressed over the number of eggs recorded over the entire dataset (2013–2023)	42
Figure 16. Scatterplot of monthly rainfall (mm) regressed over measured water depth at sites where egg clumps were recorded over the entire dataset (2013–2023).	42
Figure 17. Tile plot of attachment surface distributed over every month in which egg clumps were recorded over the entire dataset (2013 -2023).	43
Figure 18. Tile plot of developmental stage of egg clumps distributed over every month in which egg clumps were recorded over the entire dataset (2013–2023).	44
Figure 19. Tile plot of shade level recorded over every month of the entire dataset (2013–2023).	45
Figure 20. Time-series plot depicting Gosner Stage progression over time for the February 2024 egg clump isolation experiment. Desiccation and predation events are depicted with asterisks and a cross, respectively for the egg clump isolation treatment.	59
Figure 21. Time-series plot depicting Gosner Stage progression over time for the February 2024 egg clump isolation experiment. The control group depicts hatching events with a plus symbol	60
Figure 22. Time-series plot illustrating Gosner Stage progression over time for the April 2024 egg clump isolation experiment. The triangle symbol represents a desiccation event for the egg clump moistening treatment	61
Figure 23. Time-series plot illustrating Gosner Stage progression over time for the April 2024 egg clump isolation experiment. The triangle symbol depicts a desiccation event and the square symbol represents a predation event for the egg clump isolation treatment.	62

Figure 24. Time-series plot illustrating Gosner Stage progression over time for the April 2024 egg clump isolation experiment. A predation event is represented by a square for the control treatment.....	63
Figure 25. Power curve of egg clump isolation (ECI) and control treatments of the February 2024 egg clump isolation experiment, illustrating developmental rate (Gosner Stage) over time (Days Since Monitoring Started)	66
Figure 26. Power curve of egg clump moistening (ECM) treatment, egg clump isolation (ECI) treatment and control treatment from April 2024 egg clump isolation experiment illustrating developmental rate (Gosner Stage) over time (Days Since Start).....	67
Figure 27. Call profile of the "Click" call type, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram	68
Figure 28. QR code linking to audio of the “Click” call type	68
Figure 29. Call profile of the "Croak" call type, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram	69
Figure 30. QR code linking to audio of the “Croak” call type	69
Figure 31. Call profile of the "Distress call" call type, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram	70
Figure 32. Call profile of the "Distress call" call type, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram	71
Figure 33. QR code linking to audio of the “Distress call” call type	71
Figure 34. Call profile of the "Squeak" call type, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram	72
Figure 35. Call profile of the "Squeak" call type, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram	72
Figure 36. Call profile of the "Squeak" call type, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram	73
Figure 37. QR code linking to audio of the “Squeak” call type.....	73

Figure 38. Call profile of the "Squeak-trill" call type, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram	74
Figure 39. Call profile of the "Squeak-trill" call type, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram	75
Figure 40. QR code linking to audio of the "Squeak-trill" call type.....	75
Figure 41. Call profile of the "Trill" call type with 13 individual notes, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram.....	76
Figure 42. Call profile of the "Trill" call type with three individual notes, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram	76
Figure 43. Call profile of the "Trill" call type with 13 individual notes, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram.....	77
Figure 44. QR code linking to audio of the "Trill" call type.....	77
Figure 45. Call profile of the "Two-click" call type, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram	78
Figure 46. QR code linking to audio of the "Two-click" call type.....	78
Figure 47. Bar graph of total number of calls detected throughout each month from April 2023 to April 2024	81
Figure 48. Dot plot of call counts per hour with a blue line indicating the summary of calls for each hour, and a red line as a smoothed approximation of the blue summary line.....	83
Figure 49. Scatterplot of total calls detected per month vs monthly rainfall (mm) fitted with a regression line	84
Figure 50. Scatterplot of the total number of calls detected per day vs mean daily temperature (°C) fitted with a regression line ($y = 1334.6x + -3877.34$)..	85
Figure 51. Line graph of mean centre frequency (Hz) detected for calls per month	86

Figure 52. Bar graphs dividing the number of recorded visual behaviours into behavioural categories.....	89
Figure 53. Bar graph of all recorded visual behaviours employed by sexes.....	90
Figure 54. Mosaic plot of visual behaviour categories divided by sex	91
Figure 55. Mosaic plot of call types divided into vocalisation category.....	93
Figure 56. Bar graph of call types as recorded on different substrates	94
Figure 57. Tile plot illustrating visual behaviours in proportion to call types employed in multimodal communication. The legend indicates the counts of visual behaviours used with respective call types.	96

LIST OF TABLES

Table 1. Summary statistics for egg clump monitoring data collected from 2013–2023 in the VCNR. Summary statistics are provided with means and standard deviations (sd) where applicable. Variables include year, total number of egg clumps, mean number of egg clumps, total number of eggs, mean number of eggs, mean height of egg clumps (cm), water depth at recorded egg clumps (cm), total rainfall (mm), and mean rainfall (mm).....	46
Table 2. Multinomial regression model outputs for monthly rainfall vs developmental stage (2013–2017). Coefficient estimate, standard error, p-value, lower confidence interval and upper confidence interval are presented for each developmental stage	48
Table 3. Multinomial regression model output for monthly rainfall vs developmental stage (2018–2023). Coefficient estimate, standard error, p-value, lower confidence interval and upper confidence interval are presented for each developmental stage	49
Table 4. Multinomial regression model output for monthly rainfall vs attachment surface (2013–2017). Coefficient estimate, standard error, p-value, lower confidence interval and upper confidence interval are presented for each attachment surface	49
Table 5. Multinomial regression model output for monthly rainfall vs attachment surface (2018–2023). Coefficient estimate, standard error, p-value, lower confidence	

interval and upper confidence interval are presented for each attachment surface	50
Table 6. Multinomial regression model output for monthly rainfall vs attachment detail (2013–2017). Coefficient estimate, standard error, p-value, lower confidence interval and upper confidence interval are presented for each attachment detail.....	50
Table 7. Multinomial regression model output for monthly rainfall vs attachment detail (2018–2023). Coefficient estimate, standard error, p-value, lower confidence interval and upper confidence interval are presented for each attachment detail.....	51
Table 8. Multinomial regression model output for monthly rainfall vs level of shade (2013–2017). Coefficient estimate, standard error, p-value, lower confidence interval and upper confidence interval are presented for each level of shade.....	53
Table 9. Multinomial regression model output for monthly rainfall vs level of shade (2018–2023). Coefficient estimate, standard error, p-value, lower confidence interval and upper confidence interval are presented for each level of shade.....	53
Table 10. Egg clump isolation experiment: February 2024. ECI = Egg clump isolation treatment, Control = Control treatment.....	54
Table 11. Egg clump isolation experiment: April 2024. ECM = Egg clump moistening treatment, ECI = Egg clump isolation treatment, Control = Control treatment	56
Table 12. Summary statistics for egg clump isolation experiment: Experimental treatment (February 2024).....	57
Table 13. Summary statistics for egg clump isolation experiment: Control treatment (February 2024).....	57
Table 14. Summary statistics for egg clump isolation experiment: Egg clump moistening treatment (April 2024).	58
Table 15. Summary statistics for egg clump isolation experiment: Egg clump isolation treatment (April 2024).....	58
Table 16. Summary statistics for egg clump isolation experiment: Control treatment (April 2024).....	58

Table 17. Ordinal logistic regression model results for Gosner Stage (February 2024). Predictor variables for both egg clump isolation (ECI) and control groups are presented with estimates and p-values.	64
Table 18. Ordinal logistic regression model results for Gosner Stage: Egg clump moistening treatment (April 2024). Predictor variables are presented with estimates, standard errors and p-values.	64
Table 19. Ordinal logistic regression model results for Gosner Stage: Egg clump isolation treatment (April 2024). Predictor variables are presented with estimates, standard errors and p-values	65
Table 20. Ordinal logistic regression model results for Gosner Stage: Control treatment (April 2024). Predictor variables are presented with estimates, standard errors and p-values.	65
Table 21. Identified call types and number of calls for each call type used for spectral measurements and analyses	68
Table 22. Call parameters (means and standard deviations) of different call types.....	79
Table 23. Band-Limited Energy Detector output from the Raven Pro Batch Detection tool. Outputs were organised in monthly categories with mean temperature (°C), total call counts per month (n) and monthly rainfall (mm).....	80
Table 24. The number of calls detected over 24 hours (mean and standard deviation) with mean temperature (°C).	81
Table 25. Behavioural category and number of times each category was recorded for each sex.....	87
Table 26. Visual behaviours recorded for each sex, describing the context in which these behaviours were recorded for each sex.	87
Table 27. Substrates on which visual behaviours were recorded.....	89
Table 28. Visual behaviours observed to be used conjunctively, the sex which employed the behaviours, social contexts in which behaviours occurred and the behavioural categories. The social context category shows which sex facilitated the dual behaviour on which other sex or individual/s.....	91

Table 29. Call types identified during field observations	92
Table 30. Behavioural category of call types observed in the field	92
Table 31. Substrates on which calling occurred at the study site, identified from video recordings.....	93
Table 32. Multimodal behaviours (call type emitted with visual behaviours) recorded through field observations. 'n' is the total number of times a call type was recorded with the corresponding visual behaviours, and 'n2' is the number of times a specific visual behaviour is recorded with the corresponding call type.....	95
Table 33. Behavioural context identified for each call type employed in observed multimodal interactions	97
Table 34. Number of individuals engaged in each recorded multimodal interaction, with the number of occurrences	97
Table 35. Substrate on which multimodal behaviours were recorded.	97

Chapter 1: Introduction

1.1 Global amphibians in decline

In an era marked by escalating environmental challenges and the alarming loss of biodiversity, critical population ecology and habitat conservation research into the often-overlooked realm of amphibian conservation is vital (IUCN SSC Amphibian Specialist Group 2024). Amphibians play an integral part in the global ecological tapestry - serving as both sentinels of environmental health and indicators of ecosystem integrity (Valencia-Aguilar et al. 2013) - and are facing unprecedented threats that demand immediate attention and targeted conservation (Campbell Grant et al. 2023; IUCN SSC Amphibian Specialist Group 2024).

Amphibians are found on every continent except Antarctica. Of the 8 847 species described to date (Frost 2025), the majority are distributed in humid tropical environments around the globe (Luedtke et al. 2023). Three Orders of amphibians are recognized: Anura (frogs and toads), Caudata (salamanders and newts) and Gymnophiona (caecilians). Order Anura is the most specious Order in Class Amphibia, with 7 794 species described worldwide (Frost 2025), comprising 88% of global amphibian diversity (Luedtke et al. 2023). Anurans are the only amphibians in South Africa, where this study took place (Du Preez and Carruthers 2017).

A worldwide decline in amphibian populations has been reported since the 1970's (Stuart et al. 2004, Secretariat of the Convention on Biological Diversity 2020, Luedtke et al. 2023). According to a report on the state of the world's amphibians (SOTWA) led by Re:wild, Synchronicity Earth and the IUCN SSC Amphibian Specialist Group, which was based on the recent second Global Amphibian Assessment (GAA2), 40.7% of amphibians worldwide are globally threatened (Luedtke et al. 2023). Of all presently described amphibian species 92.9% (8011) have been assessed for the IUCN Red List (IUCN 2024). Globally threatened amphibian species are classified under the IUCN Red List of threatened categories, namely Vulnerable, Endangered and Critically Endangered (IUCN 2024). Documented amphibian extinctions continue to increase, with 37 amphibian species being confirmed extinct since 1980 (Luedtke et al. 2023). The number of known amphibian extinctions may be as many as 222 species over the last 150 years, owing to the number of species in the Critically Endangered (CR) category being tagged as Possibly Extinct (CR(PE)). The number of species in this group as of 1980 was 24, increasing to 162 in 2004, and rising to 185 in 2022 (Luedtke et al. 2023). Habitat loss and degradation are the most important threats to the survival of amphibian populations. Worldwide statistics show that 77% of threatened amphibian species are negatively impacted by agriculture, 53% by plant harvesting and timber production, and 40%

by the development of infrastructure (Luedtke et al. 2023). Amphibians globally are declining more rapidly and are more threatened than any other vertebrate Class, including in South Africa (Stuart et al. 2004; Measey 2011; Skwono et al. 2019). The most prevalent threats in southern Africa include habitat destruction resulting from urbanization, crop farming, invasive alien vegetation, afforestation and wetland drainage (Minter et al. 2004; Measey 2011). The consistency between patterns of major threats which affect amphibian populations has been highlighted by Red List assessments, both globally and in South Africa. The most notable threats are aquacultural and agricultural activities, which are coupled with biological invasions in South Africa (Measey 2011). In an approach used by Mokhatla et al. (2012), alien invasive plants and land transformation have also been identified as significant threats, over the long term, to the breeding success of stream-breeding and terrestrial-breeding frogs in South Africa. Their approach focused on assessing species at a sub-continental scale (QDS resolution), instead of the single species scale as used by Red List assessors, emphasizing the relationships between different breeding strategies of frogs and the anthropogenic threats they face. This approach provides another perspective for amphibian conservation where the vulnerability of frogs to their environment is assessed at their various life stages, which may better inform protection efforts.

Direct threats include anthropogenic processes or activities that have resulted in, are presently inducing, or could potentially culminate in, the inability to achieve biodiversity objectives (Salafsky et al. 2008; Measey 2011). Six strategic objectives are outlined in the National Biodiversity Strategy and Action Plan, namely: (1) management of biodiversity assets and their contribution to the economy, rural development, job creation and social well-being is enhanced, (2) investments in ecological infrastructure enhance resilience and ensure benefits to society, (3) biodiversity considerations are mainstreamed into policies, strategies and practices of a range of sectors, (4) people are mobilised to adopt practices that sustain the long-term benefits of biodiversity, (5) conservation and management of biodiversity is improved through the development of an equitable and suitably skilled workforce, and (6) effective knowledge foundations, including indigenous knowledge and citizen science, support the management, conservation and sustainable use of biodiversity (Government of South Africa 2015). Focusing on direct threats guides researchers to establish actions that, if reversed, are most likely to contribute to the improved conservation of threatened species (Salafsky et al. 2008). Direct threats may be driven by political, social, economic, institutional, or cultural factors. These factors either enable or contribute to the presence or continuity of immediate direct threats, often increasing the extinction risk to species (Salafsky et al. 2008; Measey 2011).

Chytridiomycosis, caused by two fungal species, *Batrachochytrium dendrobatidis* and *B. salamandrivorans*, is a direct and immediate threat which has been unambiguously linked to declines in amphibian species and has been described as responsible for “the greatest recorded loss of biodiversity attributable to a disease” (Scheele et al. 2019; Lambert et al. 2020; IUCN SSC Amphibian Specialist Group 2024). Even though declines related to chytridiomycosis have led to many amphibians disappearing globally, the precise number of species affected by this wildlife disease remains controversial. Many cryptic, or less well-understood threats to amphibians exist and quantification of these threats at the different life stages of amphibians may be difficult (Measey 2011).

Climate change may pose a significant threat to amphibian populations and their distributions and densities (Blaustein and Kiesecker 2002; Measey 2011; Miller et al. 2018), impacting them both directly and indirectly, especially in areas where natural habitats have been modified, polluted or fragmented, or where pathogens are prevalent (Minter 2011; Miller et al. 2018; Campbell Grant et al. 2023). Climate change has been identified as one of the most important emerging threats to amphibians (Campbell Grant et al. 2023; Luedtke et al. 2023). The human population’s resource requirements, growth and size, which ultimately lead to climate change, are the foremost factors responsible for the observed changes in the phenology, species range shifts and increased rates of spread of infectious diseases in amphibians (Vitousek et al. 1997; Mokhatla et al. 2012; Miller et al. 2018). Direct threats that climate change poses to amphibians are tied to fluctuations in temperature (extreme cold or heat) and precipitation (irregular rainfall or drought patterns), both of which are critically important factors of physiological tolerances in amphibians (Hillman et al. 2008; Blaustein et al. 2010; Minter 2011; Miller et al. 2018; IUCN SSC Amphibian Specialist Group 2024). When these tolerances are exceeded, mortalities occur (IUCN SSC Amphibian Specialist Group 2024). Climate change also indirectly affects amphibian populations in many ways, where quantifiable changes can be observed in amphibian developmental rate, breeding timing, metamorph dispersal, disease dynamics, immune function, exposure to elevated UV-B radiation levels, ecological factors including habitat loss or modification (hydrology, soil and vegetation), predator-prey relationships and food availability, competition within communities and reduction in genetic diversity and fitness (Middleton et al. 2001; Carey and Alexander 2003; Alan Pounds et al. 2006; Allentoft and O’Brien 2010; Blaustein et al. 2010; Minter 2011; Miller et al. 2018; Luedtke et al. 2023; IUCN SSC Amphibian Specialist Group 2024). Synergisms are believed to exist between all these effects and are often convoluted and rarely understood (Veith et al. 2004), contributing to the need for research on underlying factors driving extinction risk in species. Extinction risk of a species due to climate change depends on the species’ exposure and sensitivity to changed climatic variables (Dickinson et al. 2014).

Exposure is determined mainly by the anthropogenic threats experienced by a species at a geographic position (e.g. habitat loss) and is defined as the average predicted change in measurable climate variables at these geographic positions across a species' extent of occurrence (extrinsic factors), whereas the ability of a species to tolerate these changes in climate variables is its sensitivity (intrinsic factors) (Williams et al. 2008; Dawson et al. 2011; Dickinson et al. 2014). Elevated extinction risk for species in a particular region is not necessarily caused by substantial magnitude of changes in climate of that region alone. However, an interplay of high exposure, high sensitivity and a species' adaptive capacity to climate change are expected to be intimately linked to a species' risk of extinction (Dawson et al. 2011; Dickinson et al. 2014). Highly sensitive species may still experience declines in regions where exposure to climate change is low, placing an emphasis on understanding the drivers of extinction risk and underlying patterns of exposure and sensitivity of species in reaction to climate change, rather than solely on patterns of predicted climate change (Williams et al. 2008). Adjusting the focal point of conservation interventions to account for species exposure and sensitivity to climate change could greatly benefit the management of regions and species at high risk of extinction. Measey (2011) suggests that threat identification should be an integral part of any research conducted on amphibians, especially in southern Africa, where differentiation and determination of threats and contributing factors should be based on quantitative research, wherever viable. Amphibian declines (excluding chytridiomycosis) may not be attributable to any single causative factor, but rather to several, potentially dynamic, interactions between biotic and abiotic components in any given habitat (Blaustein and Kiesecker 2002).

1.2 South African amphibians in decline

There are 180 described species of frogs in southern Africa, and 140 of these occur in South Africa (Frost 2025). The two main areas of frog endemism in South Africa are the Western Cape and KwaZulu-Natal provinces (Measey et al. 2019). In the same year that the first Global Amphibian Assessment was completed (Stuart et al. 2004), the Atlas and Red Data Book of the Frogs of South Africa, Lesotho and Swaziland, was published (Minter et al. 2004). The Southern African Frog Atlas Project (SAFAP) has served as a key instrument for assessing South Africa's amphibians in need of further conservation research (Measey et al. 2019). The aims of SAFAP were sevenfold: utilise quarter-degree grid cells of southern Africa to compile a comprehensive list of frog species occurring in each; map the distributions of all frog species occurring in the atlas region; use the SAFAP data to assess the conservation status of these frog species; create a database to inform biological research and conservation planning for

southern African frogs, and finally, to raise public awareness and appreciation of frogs and their conservation needs (Minter et al. 2004).

Declines in southern African amphibians have been observed only at the local population level, where these declines are usually confined to those areas which are directly influenced by relevant threats (Minter et al. 2004). There are very few studies in South Africa that include datasets long-term enough to provide quantifiable information on amphibian population dynamics, including possible declines in their distributions and population sizes (Measey et al. 2019).

1.3 Monitoring in amphibian conservation

Conservation prioritization for species and areas is increasingly important to begin to address amphibian population declines. Community-level monitoring helps identify species, or groups of species, which are susceptible to declines, which in turn could provide perspectives on habitat change (Veith et al. 2004). A crucial aspect in understanding trends within amphibian populations is the regular monitoring of these populations. Monitoring also provides a means of evaluating conservation actions. Most amphibians exhibit natural fluctuations in their population numbers, which necessitates long-term monitoring datasets to accurately discern trends over time (Marsh 2001; Measey 2011). The collected monitoring data can be used not only to determine population trends, but also to evaluate the effectiveness of conservation interventions and other land management practices. It is advisable for monitoring techniques to be quantitative, involving abundance estimates, with confidence intervals for meaningful comparisons. Careful consideration should be given to selecting monitoring sites and methods that can be realistically sustained over extended periods with consistent approaches (Measey 2011).

Monitoring that supports conservation management has three main objectives: 1) to provide information on when the system departs from the preferred or baseline state, 2) to reveal the effects of disturbances and perturbations, and 3) to quantify management action successes or failures, and therefore monitoring should inform adaptive management (Legg and Nagy 2006). Global declines in amphibians are undeniably occurring, however, difficulty remains in establishing whether declines are caused by natural fluctuations and variability of populations, or whether declines can be attributed to anthropogenic causes (Gibbons et al. 2000). Most field studies lack the consistency, or duration, in which conclusive evidence can be found to indicate whether declines are due to normal fluctuations or “unnatural” causes (Gibbons et al. 2000). Therefore, short-term monitoring may only provide insights into the

present status of population structures and fluctuations, which emphasises the importance of long-term monitoring programs to detect and determine the direction of significant trends in population health or size (Gibbons et al. 2000; Measey 2011). Habitat management practices, and the effects of conservation actions on particular species or populations, can be assessed through long-term monitoring - given that the data are quantitative, reliable and repeatable over the long-term (Legg and Nagy 2006; Measey 2011). Research efforts have focused on forming a clearer picture of the proximate causes of these declines, and which roles limiting factors play in these declines (Minter et al. 2004). From these research efforts, numerous monitoring schemes have been initiated as early warning systems (Measey et al. 2017). Inadequate statistical power, loss of the integrity of long-term monitoring data records, and loss of personnel are key contributing factors to the failures of some long-term monitoring schemes (Measey et al. 2017).

The selection of methods for designing monitoring protocols should find an optimal balance between practicability, specific objectives, efficiency and environmental impact (Rödel and Ernst 2003). As conservation managers strive to implement landscape-scale monitoring with limited financial resources, statistical power is often compromised. In this context, optimisation of survey design efficiency becomes crucial. Optimal survey design aims to maximise statistical power or minimise financial costs considering constraints defined by management goals, budgets, and system-specific characteristics (Field et al. 2005). Essential prerequisites involve the capability to measure and quantify detectability and assess the costs associated with various survey frameworks. These factors should be integrated into analyses that evaluate trade-offs between survey configurations that enhance statistical power and reduce financial costs (Field et al. 2005). In reality, declines will not unfold in a linear manner. Instead, they will be influenced by multiple stochastic factors that interact to produce irregular dynamics typical of natural populations (Field et al. 2005). It becomes significantly more complicated to detect trends under these circumstances. The most effective monitoring design for a particular species inevitably relies on the unique characteristics of its habitat and ecology, as well as the organisation conducting the surveys (Field et al. 2005). Instead of arbitrary statistical conventions, management objectives should ultimately determine the conclusions drawn from data and previous management actions (Field et al. 2005). More considerations for designing monitoring protocols as outlined by Measey (2011) include the importance of incorporating diverse sampling techniques and conducting temporal and seasonal sampling. Also advisable is to ensure consistency among observers to reduce sampling error. This may involve training multiple observers who work in pairs to retain uniformity. Long-term monitoring should also account for the replacement of personnel due to job changes or retirement. Flexibility in sampling is crucial for capturing a diverse range of anuran life-history stages

through which accurate identification and measurements can be done. Detectability can be influenced by various factors such as weather, moon brightness, precipitation, and prey availability (MacKenzie et al. 2002; Measey 2011). A recommendation is to position monitoring sites near national weather stations or install a weather monitoring station alongside a monitoring site. Accessibility is another key consideration, especially since sites may need to be visited during inclement weather when vehicular access is likely to be challenging. Lastly, effective communication of monitoring results to both the wider research community and the general public is emphasised as an integral part of the monitoring process.

Exploring fundamental aspects of biology, behaviour and ecology can be both time-consuming and costly, yet is often essential for constructing effective conservation strategies (Measey 2011). Employing a comprehensive strategy that integrates various techniques in active or passive sampling, such as genetics, radio-telemetry, camera trapping, acoustic monitoring, niche modeling, and ecological characterisations, is likely to establish a robust foundation for interpretation (Agha et al. 2015; Hobbs and Brehme 2017; Desjonquères et al. 2020). Estimation of population size may be crucial for determining extinction risk (Measey 2011; Desjonquères et al. 2020).

1.4 Bioacoustics and acoustic monitoring

The field of bioacoustics has experienced significant research focus, drawing insights primarily from ecology, behavioural sciences, evolution and physiology (Toledo et al. 2015; Köhler et al. 2017). Various sounds are produced by animals, which convey some form of signal to conspecifics or to potential predators – these signals are highly valued in taxonomic research (Köhler et al. 2017). A signal is characterised as the specialised, species-specific morphology or behaviour which influences the behaviour of another individual, either in the present or in the future (Owren et al. 2010; Köhler et al. 2017). Amphibian advertisement calls are those calls typically heard in the natural environment, and these calls seem to fill two primary purposes - attracting potential mates and conveying information on territory to conspecifics (Köhler et al. 2017). Mate choice and recognition in amphibians may also involve pheromones, visual signalling, for example, inflation of often colourful vocal sacs, foot waving, surface vibrations, acoustic signals, and in some species, multimodal communication using different combinations of these mechanisms (Toledo et al. 2007; Boeckle et al. 2009; Köhler et al. 2017). Anuran calls are emitted in a wide range of contexts. These vocalisations are subdivided into three encompassing categories, with each having various subcategories: aggressive, defensive and reproductive calls (Toledo et al. 2015).

Acoustic signalling plays a vital role in the life-histories of most anurans, where information is transmitted or received which drives fundamental processes crucial to survival and persistence of species and communities. Acoustic patterns and performances of animals are intimately tied to animal biomass, dynamic relationships between the energetic environment, and the structure of social interactions, indicating that these acoustic patterns and individual acoustic performance may be sensitive to environmental conditions such as humidity, temperature, noise and social organisation (Krause and Farina 2016). Acoustic signalling may also be coupled to visual signalling in certain anuran species, inducing multimodal communication between conspecifics (Caldart et al. 2014). Investigating the acoustic phenology in frog communities gives insight into the changes of the interspecific relationships and physiological rhythm that take place, between or in organisms, as a response to their environmental cues or constraints (Krause and Farina 2016).

Auditory monitoring techniques, such as passive acoustic monitoring (PAM), have gained traction as non-invasive bioacoustic methods to monitor and sample ecosystems. PAM involves the recording and analysis of sounds produced within the natural environment, to derive information on the presence of specific species or the overall dynamics and conditions of an ecosystem (Desjonquères et al. 2020). This technique is used to acquire estimates of population size or the number of calling males within an area. Automated recording systems (ARS) provide a permanent record of sampling occasions that can be interpreted later, allowing collection of call data without observers being present and allowing data to be collected by staff who are unskilled in identifying calls that are species-specific (Measey et al. 2017). For a range of ecological applications, including the identification of invasive, rare or threatened species, as well as contrasting the variations in the health and structure of an ecosystem, and the continuous monitoring of environments, PAM can be both highly precise and cost-effective (Desjonquères et al. 2020). PAM has good potential for use in environments that are of low visibility, such as dense forests, as sound propagation is not as strongly affected by obstacles as other sensing methods, such as visual detection. Sounds produced during mating, territorial disputes or distress conditions can be detected through PAM (Desjonquères et al. 2020). In the context of endangered species, species-specific sounds can be used to survey and investigate various aspects of their population dynamics (Desjonquères et al. 2020). Temporal patterns in calling schedules of species have been observed at the species and community levels, both in the diurnal as well as seasonal scales, indicating the importance of creating a recording schedule aimed at answering specific ecological questions (Köhler et al. 2017; Desjonquères et al. 2020). Sampling acoustic diversity in freshwater ecosystems at short, regular intervals, multiple times a day may be more efficient in comparison with longer recordings over longer intervals (Linke et al. 2020). Freshwater environments are subjected to

fast declines in biodiversity, at rates far greater than other threatened environments (Dudgeon et al. 2006). Some of these changes in biodiversity are detectable in the soundscapes of these environments (Pavan, 2017), making acoustic monitoring particularly suitable. Regular acoustic surveys may yield time series, which can reveal trends and effects of short-term global changes (Krause & Farina, 2016). Ectothermic species and threatened species may be sensitive to global warming. These sensitivities can be observed in many call parameters of frog species and other ectotherms. Llusia et al. (2013) have shown that the chorus activity of *Hyla molleri* and *H. meridionalis* are influenced by air temperature and relative humidity, showing that PAM, in conjunction with environmental data, can be used as a tool for detecting changes in anuran behaviour resulting from changes in climatological conditions.

Any long-term comparison in an unstable environment should take changes in habitat into careful consideration, especially when monitoring is conducted in the same areas over time (Heinermann et al. 2015). Forest regeneration, as well as degradation, can occur over time, and alterations in community composition may be attributed to these changes in habitat, rather than factors that the monitoring effort aims to capture - such as climate change or the effects of pathogens (Heinermann et al. 2015). Nonetheless, monitoring plays a crucial role in assessing whether amphibian populations reflect the impacts of climate change, and utilising models becomes instrumental in determining optimal locations for focal monitoring of frog species or communities which may be affected by climate change to any extent (Measey 2011). To account for local spatial environmental effects, it is important to implement monitoring across a variety of habitats and altitudes (Measey 2011).

According to Heinermann et al. (2015) (citing Feinsinger, 2001), surveying megadiverse areas presents a challenge in field ecology because of the often impractical or economically restrictive sampling efforts required to achieve the desired statistical power. For studies aiming to uncover long-term trends, accurate designs are essential. Data contributed by volunteers, commonly known as 'citizen scientists', can be valuable if there is a predefined ultimate goal and a known analysis approach (Measey 2011). Local people possessing specialised knowledge about the amphibian diversity in their native areas act as para-ecologists, making valuable contributions to the wider scientific community (Heinermann et al. 2015). Given adequate support and training, along with a comprehensive understanding of the basic activity patterns of local anurans, it appears feasible to establish additional long-term programs in other important areas of the country. Here these programs would serve to monitor potential declines or extinctions, enabling rapid implementation of conservation measures if needed (Heinermann et al. 2015).

1.5 Challenges of amphibian conservation in South Africa

In South Africa, there are severe resource and capacity constraints in national and provincial reserves (Measey 2011). These constraints are also evident in academic and institutional amphibian conservation organisations across the country (Measey et al. 2019). There is an absence of dedicated funding for the initiation and sustained maintenance of new long-term monitoring sites, as ongoing monitoring of threatened species is limited to the short-term funding cycles of academic institutions or is funded by the researchers themselves (Measey 2011). Further research is necessary to assess the effectiveness of existing monitoring techniques. This requires financial investment in infrastructure, such as linking recording equipment to weather stations at existing sites as well as investment in the development of techniques that are tailored to the unique characteristics of some species (Measey 2011).

The term “capacity” in the context of the Convention on Biological Diversity includes “the training of, and knowledge exchange between, people and the strengthening of institutions” (Korn et al. 2004; Schmeller et al. 2017). Therefore, capacity-building encompasses a broad spectrum of activities, which include conducting training workshops and conferences, organising workshops, developing and distributing publications, securing funding, facilitating data management and sharing, and sourcing technology which enhances the capabilities of governmental and non-governmental organisations and research institutions (Schmeller et al. 2017). Motivated citizens can assist in gathering biodiversity data through both extensive (citizen science), and intensive monitoring systems (para-ecologists), which would serve to expand the participation of local communities and the general public, increasing the cost-effectiveness of monitoring (Schmeller et al. 2017). In 2019, only nine people were employed or working at least part time on amphibian conservation. However, a substantial increase was noted in the number of postgraduate students (MSc and PhD) pursuing studies on amphibians in South Africa (Measey et al. 2019). Amphibians are not particularly popular amongst the South African public, where a large proportion of people view frogs in an apathetic or negative light, often unaware of the threats confronting South African anurans or the value frogs have in ecosystems (Tarrant et al. 2016; Phaka et al. 2022; Phaka et al. 2023). Publications revolving around amphibians which are popular are arguably the most significant contribution to capacity building in South Africa. These publications play a vital role in contributing to Citizen Science, which, in turn, benefits both prospective citizen scientists as well as individuals considering careers in herpetology (Measey et al. 2019). Popular publications include frog field guides (Du Preez and Carruthers 2017; Channing 2001; Channing and Rödel 2019), a frog field guide written in an ethnic African language (Phaka et al. 2017), a children’s book (Tarrant 2021), and an electronic application (Measey et al. 2019). Involving local communities and volunteers in monitoring is crucial to maximize sampling effort

through which a broader picture of biodiversity status and environmental change can be acquired (Schmeller et al. 2017). Additional advantages of engaging local communities in biodiversity monitoring include a simultaneous rise in environmental awareness, trust in policy outcomes supported by the gathered observational data, and improved management practices because of their active participation (Schmeller et al. 2017).

1.6 *Natalobatrachus bonebergi*: conservation, life-history information and study objectives

Through the Ezemvelo KZN Wildlife Norms and Standards (Goodman 2011), detailed monitoring and surveillance protocols have been developed for KwaZulu-Natal's threatened frog species, including the Kloof Frog (*Natalobatrachus bonebergi*), for which a wealth of data has been collected over the past decade.

In 2013, a monitoring and surveillance protocol for KwaZulu-Natal's (KZN) threatened frog species was developed by the Endangered Wildlife Trust's Threatened Amphibian Programme (EWT-TAP) and Ezemvelo KZN Wildlife (EKZNW) (Tarrant and Armstrong 2013). The methodology for monitoring *N. bonebergi* was tested by Ezemvelo Honorary Officers at the Vernon Crookes Nature Reserve (VCNR) between December 2013 and December 2017 during which time transects were carried out monthly and thereafter took place biannually in April and December. This monitoring programme stemmed from the need to gather data to improve the understanding of population trends and responses of populations to management strategies at priority sites where threatened KZN species occur. The processes that govern immigration, emigration and natural extinction of South African frogs are poorly understood, especially for cryptic and threatened species (Tarrant and Armstrong 2013). To this end, monitoring of *N. bonebergi* using unique aspects of its breeding biology serves to gather data on these dynamics. The VCNR occurs within the Maputaland-Pondoland-Albany biodiversity hotspot – one of the only two such hotspots in Southern Africa. The VCNR is situated in the eastern central region of this hotspot, standing as a key protector of critical ecosystem services and overall biodiversity amidst urban development, sugarcane plantations and rural expansion in KZN (Critical Ecosystem Partnership Fund 2010; Bailey et al. 2016). This provincial Nature Reserve is managed by EKZNW, and data collection is facilitated by Honorary Officers of EKZNW, and by volunteers and citizen scientists, including school groups, at other sites. *Natalobatrachus bonebergi* egg clump monitoring data have been collected in the VCNR since December 2013, and has since extended to include other sites, such as Adams Mission, Crowned Eagle Estate, as well as four sites in the Eastern Cape province, namely, Hluleka-,

Silaka-, Cwebe- and Dwesa Nature Reserves (Tarrant and Armstrong 2013). For each of these sites, monitoring consists of egg clump counts along riverine transects of between 50 m and 150 m in length using an electronic application (ArcGIS Survey123, ESRI, Redlands, California, USA), which aims to provide indications of reproduction during the sampling period, thereby gauging population trends for *N. bonebergi* in the long term.

Natalobatrachus bonebergi (Hewitt and Methuen, 1913) is a monotypic species in the genus *Natalobatrachus* of the Family Pyxicephalidae (Frost 2025). It is a semi-arboreal forest specialist and prefers a habitat of slow-flowing streams interlaced with shallow pools in densely forested ravines, where it is found in dark kloofs and rocky streambeds in closed canopy environments; it has never been recorded outside shaded areas (Kok and Seaman 1989; Minter et al. 2004; IUCN 2016; Du Preez and Carruthers 2017). Female *N. bonebergi* are on average larger than males, reaching a recorded maximum snout-vent length of 37 mm (Kok and Seaman 1988), whereas the recorded maximum snout-vent length of males is 24 mm (Du Preez and Carruthers 2017). Males have conspicuous T-shaped nuptial pads on their fingers, and the vocal sacs are not well-defined, bulging only slightly when calling (Du Preez and Carruthers 2017). Males call with an inconspicuous and soft click or “chick” at irregular intervals, often from elevated positions devoid of vegetative obstructions near water (Wager 1965; Lambiris 1989, Du Preez and Carruthers 2017). This advertisement call has a recorded dominant frequency of 3.6 kHz (Channing 2001); however, little knowledge exists on the additional call types and call groups that this species employs in reproductive, aggressive or defensive behavioural contexts. *Natalobatrachus bonebergi* is an Evolutionarily Distinct and Globally Endangered (EDGE) species (Safi et al. 2013), and was first listed as Endangered in 2001 (IUCN 2023) under the B2ab(ii,iii) criteria of the IUCN because of the threats faced by this species, its severely fragmented distribution and relatively small Area of Occupancy (AOO) of about 188 square kilometres (Minter et al. 2004; IUCN 2016). The overall population size of *N. bonebergi* is declining due to several threats (Measey 2011; IUCN 2016). Deforestation, urbanisation, agricultural clearing, pollution, and siltation threaten all developmental stages of this species (Measey 2011; IUCN 2016).

Natalobatrachus bonebergi has an extended breeding season from October to May, and lay their eggs in gelatinous masses of 75 – 108 eggs, where these egg clumps are suspended above narrow, shallow streams at heights of between 20 cm – 1.2 m above the water or streambed (Kok and Seaman 1988; Kok and Seaman 1989; Channing 2001). Tadpoles hatch after approximately 6 days, whereafter the tadpoles drop into the water below (Du Preez and Carruthers 2017). *Natalobatrachus bonebergi* females lay their eggs on various attachment surfaces, such as twigs, branches, fern fronds, leaves and rocks (Kok and Seaman 1989; Minter et al. 2004; Du Preez and Carruthers 2017), above slow-flowing sections of a

stream. Egg clumps are protected from desiccation due to the females regularly visiting the egg clumps to moisten them with liquid from their cloaca (Du Preez and Carruthers 2017). Further desiccation may be prevented by laying the eggs on substrates shaded from exposure to direct sunlight, including UV radiation. Amphibians are vulnerable to extremes in environmental factors such as air temperature, relative humidity, and rainfall which greatly influence their physiological parameters (Hoffmann et al. 2021). This vulnerability naturally extends to reproductive behaviour and development of eggs, although little evidence has been collected to determine which environmental factors influence these *N. bonebergi* parameters. Knowledge of this species' phenology is therefore of utmost importance in understanding the evolutionary and ecological processes governing their behavioural patterns, which in turn will aid in advancing its, and its habitat's, conservation management (Heinermann et al. 2015). Appropriate conservation management may be achieved through the identification and monitoring of *N. bonebergi*'s reproductive behaviour, breeding biology and early developmental stages. The present study aimed to investigate these aspects by utilising data collected from different monitoring approaches. The broad objectives of the study were as follows:

(1) Egg clump monitoring data collected in VCNR from December 2013 to April 2023 were used to statistically investigate the effects of collected covariates and monthly rainfall on the number of recorded eggs and egg clumps in R and RStudio.

(2) Egg clump isolation experiments were performed where relatively newly laid egg clumps were enclosed in mesh nets to prevent females from moistening the eggs to ascertain whether females play a major role in egg survival during embryological development.

(3) PAM data collected from an SM4 Song Meter (Wildlife Acoustics), set up at a popular *N. bonebergi* breeding site in VCNR from April 2023 to April 2024, were analysed using Raven Pro acoustic analysis software to generate call counts for different temporal scales (hourly, daily, monthly). Acoustic analyses were further facilitated using R and RStudio to elucidate information about *N. bonebergi* calling activity by investigating the influences of temperature and rainfall on this species' daily circadian rhythm, monthly calling activity and changes in centre frequency over time, relative to the number of calls recorded over time.

(4) High-quality directional recordings of various call types used by *N. bonebergi* were collected *in situ* and profiled using Raven Pro. Call types were then categorised into three behavioural categories - Aggressive, Reproductive, or Defensive - based on *in situ* observations made detailing the social contexts in which these call types were emitted.

(5) Observations of *N. bonebergi* reproductive behaviours were collected *in situ*, describing various visual, acoustic and multimodal behaviours documented throughout the

study period. These behaviours were documented in the social contexts (Aggressive, Reproductive or Defensive) in which they were employed, the substrates on which they occurred and the sex exhibiting each behaviour. These behaviours were analysed in R and RStudio to investigate potential relationships between these covariates and whether these covariates influence behaviours exhibited by *N. bonebergi*. Female parental care and predation events on adults and eggs were also recorded.

Overall, this study aimed to contribute to the body of knowledge on the reproductive behaviour of *N. bonebergi*. Highlighting factors that influence the phenology of this unique and threatened species aimed to inform and improve conservation management of this species and its habitat. This study has implications for future research directions concerning climate change, habitat modification, introductions of invasive species and further investigation of reproductive behaviours in relation to *N. bonebergi* and its habitats.

Chapter 2: Materials & Methods

2.1 Study area

All data were collected in Vernon Crookes Nature Reserve (VCNR), situated on the south coast of KwaZulu-Natal (-30.2829°; 30.5867°), South Africa, with a minimum elevation of 118 m and a maximum elevation of 588 m (Figure 1, maps.google.com). The VCNR spans 2189 ha, and encompasses extensive coastal grasslands and coastal forest habitats, which include forested ravines interlaced with a network of slow flowing streams. The VCNR is surrounded by vast fields of sugarcane (*Saccharum officinarum*), gum (*Eucalyptus* spp.) plantations and tribal lands.

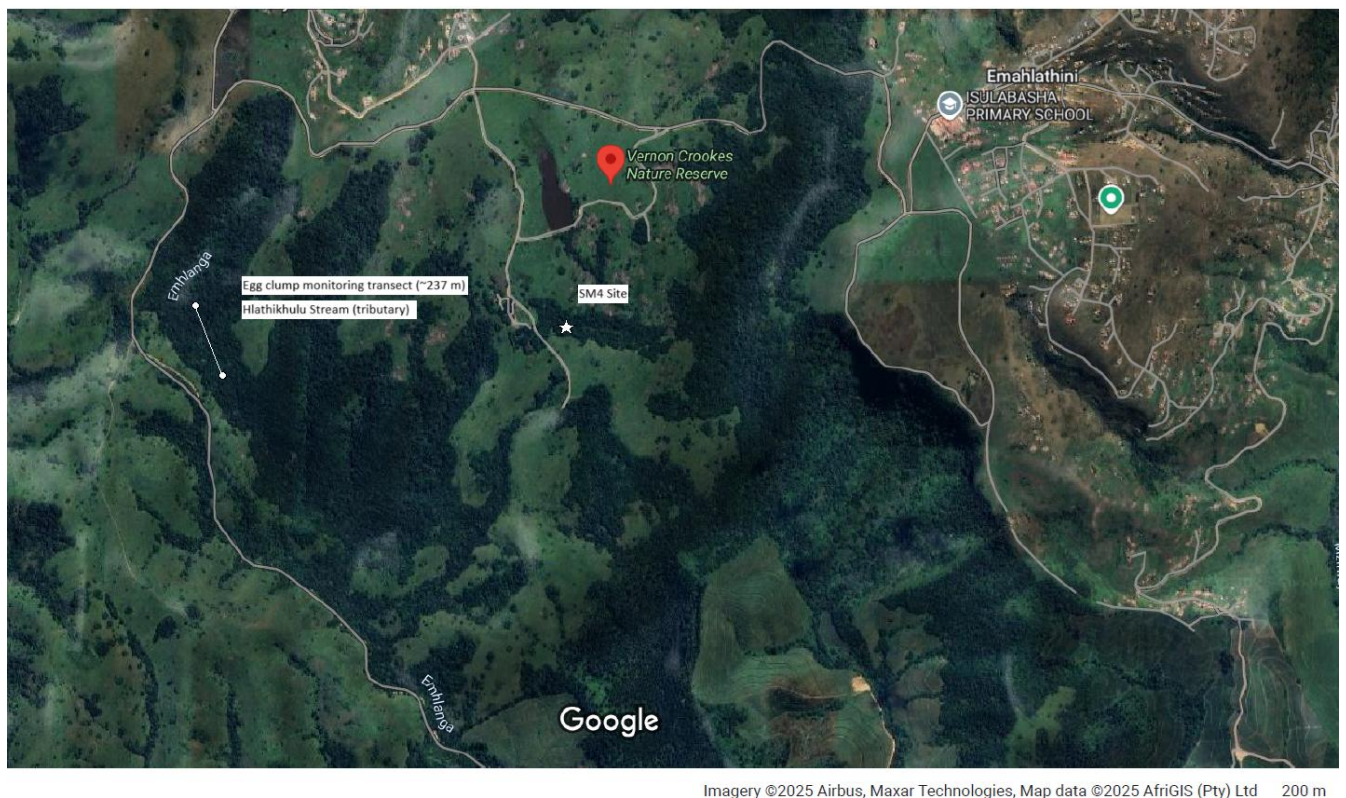


Figure 1. Map of Vernon Crookes Nature Reserve, KZN, indicating the egg clump monitoring transect (white dots connected with a line) and the SM4 PAM site (white star).

2.2 Egg clump monitoring

Applying The Endangered Wildlife Trust's Threatened Amphibian Programme (EWT-TAP) monitoring protocol (Tarrant and Armstrong 2013), a transect was identified in VCNR (start: -30.2708, 30.593, end: -30.2733, 30.594), along the Hlathikhulu Stream (tributary) spanning approximately 237 m within suitable *N. bonebergi* habitat (Figure 1). These transects

were conducted by honorary officers of Ezemvelo KZN Wildlife and members of the EWT-TAP, as well as other volunteers. Transect data were collected from December 2013 to April 2024. From 2013 to 2017, data used in this study were collected monthly to establish trends in *N. bonebergi* breeding activity throughout the year, and once trends were recognized, data collection from 2018 to 2024 was conducted biannually during peak breeding periods in December–January and in March–April and in with additional data collections done in peak breeding activity months. Quantitative data collected on *N. bonebergi* egg clumps from December 2013 to April 2023 were used for statistical analyses in R (R Core Team 2021) and RStudio (Posit Team 2023). The data analyses were divided into two parts: 2013–2017 and 2018–2024 to account for the shift from monthly data collection to biannual data collection. Descriptive statistics were obtained to summarise the data and reveal trends in egg-laying activity over the two timeframes, where additional subsets were created to further divide the data into continuous and categorical variables for ease of analyses. Linear models were constructed to investigate relationships between numerous variables collected at VCNR transect sites which may potentially relate to egg laying trends of *N. bonebergi*. Furthermore, analyses of the data were divided into the 2013–2017 timeframe and 2018–2023 timeframe. Linear models were then constructed for these separate timeframes in R (R Core Team 2021) and RStudio (Posit Team 2023) to investigate the effects of potential independent (predictor) variables (rainfall, height above water, water depth and shade) on potential dependent variables (number of egg clumps, number of eggs in egg clumps, height above water and water depth). Multinomial regression models were constructed to investigate the relationships of categorical variables that had a large number of levels within each variable against monthly rainfall as the predictor/dependent variable. These categorical variables tended to overfit regular linear models and yield inaccurate estimations of the data. The R packages ‘tidyverse’, ‘openxlsx’, ‘readxl’, ‘car’, ‘psych’ and ‘ggplot2’ were used in the analyses. The variables used for analyses include number of egg clumps, number of eggs in each clump (counted from viable images of egg clumps using DotDotGoose manual counting software), date, level of shade (estimated above each egg clump and categorised as “full shade”, “partial shade”, “light shade” or “full sun”), height of egg clumps above water (cm), depth of water (cm), attachment surface, attachment detail (if applicable, plants were identified to genus or species level, whether the surfaces were dead plant material, or whether leaves were small), developmental stage of egg clumps (ranging from newly laid, to hatched, drying or mouldy). Monthly rainfall (mm) data acquired from the nearest available weather station (Ellingham Estate Quarry, ~ 9.9 km from the VCNR transect sites) were used with the egg clump monitoring data on a monthly scale for further statistical analyses.

2.3 Egg clump isolation experiments

Egg clump isolation experiments (ECIE) were carried out in February and April 2024 at selected sites across the egg clump monitoring transect in the VCNR. ECIE conducted in February 2024 had two treatments. An experimental treatment, in which 12 relatively newly laid egg clumps were isolated using wire and plastic mesh to prevent from females reaching the egg clumps and perform parental care activities. The plastic mesh was fitted over a spiral wire frame constructed around the egg clumps (Figure 2). Adult female *N. bonebergi* were thus prevented from making physical contact with the eggs (Figure 3).



Figure 2. Egg clump isolation cage constructed around newly laid egg clump with wire and plastic mesh. iButton hygrometers were used to measure ambient temperature (°C) and relative humidity (%).



Figure 3. Female *N. bonebergi* attempting to reach egg clump. Swollen abdomen indicates that this female absorbed water to transport to egg clump.

The second treatment was a control treatment, in which 16 relatively newly laid egg clumps were identified and monitored without disturbing the eggs and allowing females to visit the egg clumps. ECIE experiments conducted in April 2024 had another treatment in addition to egg clump isolation ($n = 7$) and controls ($n = 7$): egg clump moistening ($n = 5$). Egg clump moistening entailed the same experimental design as the egg clump isolation treatment, where a pipette was filled with stream water and deposited over the entire isolated egg clump to crudely imitate egg clump moistening facilitated by female *N. bonebergi* during parental care.

iButton hygrometers (Analog Devices, Wilmington, Massachusetts) were set up with egg clumps used in both treatments to collect ambient temperature and relative humidity data. Egg clumps used for the control treatments were found at sites with similar microclimate conditions and heights to the experimental treatments. Variables collected for these experiments were: (Gosner, 1960) Stage of embryonic development, temperature ($^{\circ}\text{C}$), relative humidity (%), attachment surface type, height above water level and level of shade found at each egg clump site. Time-series plots and power curve plots were constructed for each treatment of both experiments to illustrate Gosner Stage development over time. Statistical analyses were conducted in R and RStudio using the packages 'tidyverse', 'readxl', 'car', 'ordinal' and 'ggplot2'.

2.4 Acoustic monitoring

One SM4 Song Meter (Wildlife Acoustics) (Figure 4) was set up at a shallow pool of slow-flowing water where high breeding activity of *N. bonebergi* was consistently detected throughout breeding seasons (Figure 1).



Figure 4. SM4 Song Meter by Wildlife Acoustics

This site was in the centre of the VCNR (-30.271198, 30.608831) and close to the warden's residence for security purposes. This SM4 was set to record 10 min per hour for just over a year (13 April 2023 to 21 April 2024) to obtain acoustic data linked to the species' calling behaviour. Acoustic files were recorded in the .wav format, containing data on day, time, temperature and the range of frequencies. These data were analysed through the acoustic analysis software Raven Pro and the statistical analysis software R and RStudio. High-quality recordings were collected using a Zoom F1 directional microphone to isolate, analyse and construct the call profiles of the various calls utilised by *N. bonebergi* during reproductive behaviours. The R packages 'tuneR' and 'seewave' were used to construct the call profiles. The spectral parameters of call profiles were then used to calibrate the Band-Limited Energy Detector (BLED) in Raven Pro's Batch Detection tool to batch-process the SM4 data collected over the entire year. The target signal parameters used for the Raven Pro BLED to batch detect *N. bonebergi* calls from SM4 PAM data are as follows: minimum frequency (1256 Hz), maximum frequency (3697 Hz), minimum duration of vocalisation (0.016 s), maximum duration of vocalisation (1.227 s), minimum separation between vocalizations (0.01). Noise filters were also applied, where the signal-to-noise ratio threshold (dB) was set to detect vocalisations 10 dB louder than background noise. An exclusion band was also applied to filter out all sounds under 1255 Hz. Selection tables were generated from the BLED, which was then analysed in R and RStudio using the 'tidyverse', 'readxl', 'lubridate', 'ggplot2', 'lme4' and 'MuMIn' packages. Monthly rainfall data collected at Ellingham Estate Quarry (~ 8.5 km from SM4 recording site) were compiled with the BLED output data. Analyses were aimed at investigating the circadian rhythms of *N. bonebergi* calls and to determine how calling behaviour shifts throughout the year in relation to recorded climatological data (temperature and rainfall).

2.5 Observations of behaviour

In situ behavioural observations on *N. bonebergi* were made at a second breeding site in VCNR, where the SM4 was set up (Figure 1, -30.271198, 30.608831). Using a combination of photographs, video recordings and written notes, field observations were quantified and compiled in Excel sheets. Field observations commenced on 11 April 2023 for four days, then for four days in June/July to observe behaviours outside of the *N. bonebergi* breeding season. Subsequently, field observations were conducted every three months for 8–14 days and concluded on 22 April 2024 following ten days of field observations. Effects of disturbance on *N. bonebergi* reproductive behaviours were minimized by waiting at least two minutes before observations were recorded to allow for normal behaviours to resume and observing behaviours from a distance of at least 60 cm. Observations on *N. bonebergi* reproductive behaviours were made daily between approximately 19:00 and 23:00. The following

parameters were recorded during behavioural observations: (1) the visual behaviour employed, (2) the sex of the individual, (3) the behavioural category of the recorded visual behaviour, (5) the substrate on which the behaviour occurred, (6) whether more than one visual behaviour is employed simultaneously, (7) call types employed during interactions, (8) social context if vocalisations are used, (9), sex of the calling individual, (10) substrate from which calling occurs, (11) the occurrence of multimodal displays, (12) the number of individuals present during display and (12) the substrate on which these displays occurred. Displays were classified as multimodal when a visual signal was employed simultaneously with a vocalisation or in short succession of one-another (Caldart et al. 2014).

The various parameters that were collected were organised into four categories: visual behaviours, dual visual behaviours, calling behaviours and multimodal behaviours. These data were analysed in R and RStudio using the 'tidyverse', 'ggplot2', 'car', 'psych', 'MuMIn', 'tinytex', 'janitor', 'ggmosaic', and 'purrr' packages. Frequency tables were constructed to summarise different variables from different sections, as most recorded parameters are categorical. Fisher's Exact Tests were conducted to investigate relationships between categorical variables and compare the overall distribution of counts across all four categories, as numerous variables had too few cell counts to constitute the use of a simpler test such as Chi-square tests.

Chapter 3: Results

3.1 Egg clump monitoring

3.1.1 Egg clump monitoring

Analyses of monitoring data collected from *N. bonebergi* egg clumps at the VCNR transect sites yielded insightful results on the egg-laying trends of this species over time. The data collected at the VCNR between 2013 and 2024 were not consistent over this timeframe, as the study design was altered halfway throughout this period. This change was made in response to identifying peak breeding periods, and to reduce monitoring effort. From 2013 to 2017, egg clump monitoring transects were conducted monthly, and from 2018 to 2023 these transects were conducted approximately biannually, with additional monitoring being done throughout the 2018–2023 period in months when volunteers were available for collecting data.

The total number of egg clumps recorded over both the 2013–2017 and 2018–2023 timeframes are graphically represented against the types of attachment surfaces recorded through those timeframes (Figures 5 and 6, respectively). It was evident that leaves were consistently the most common attachment surface for egg clumps across the entire dataset.

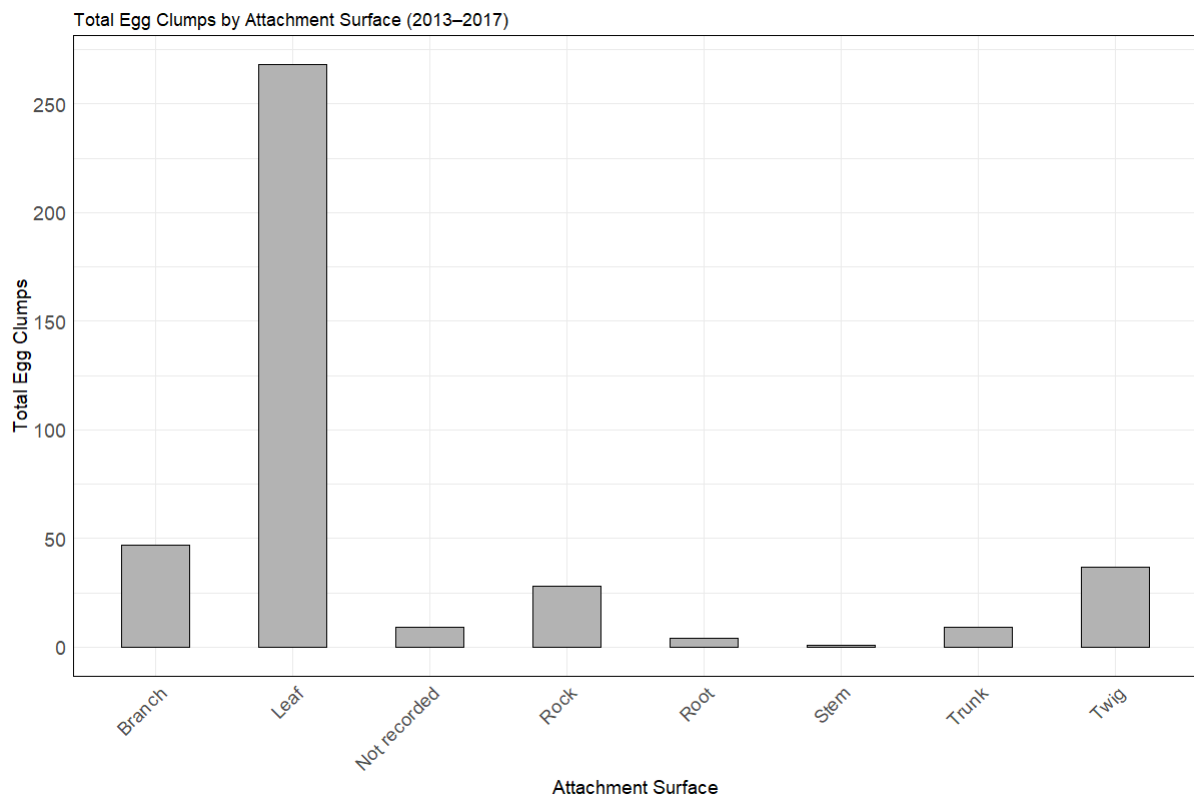


Figure 5. Bar graph of total egg clumps per attachment surface for the 2013–2017 timeframe.

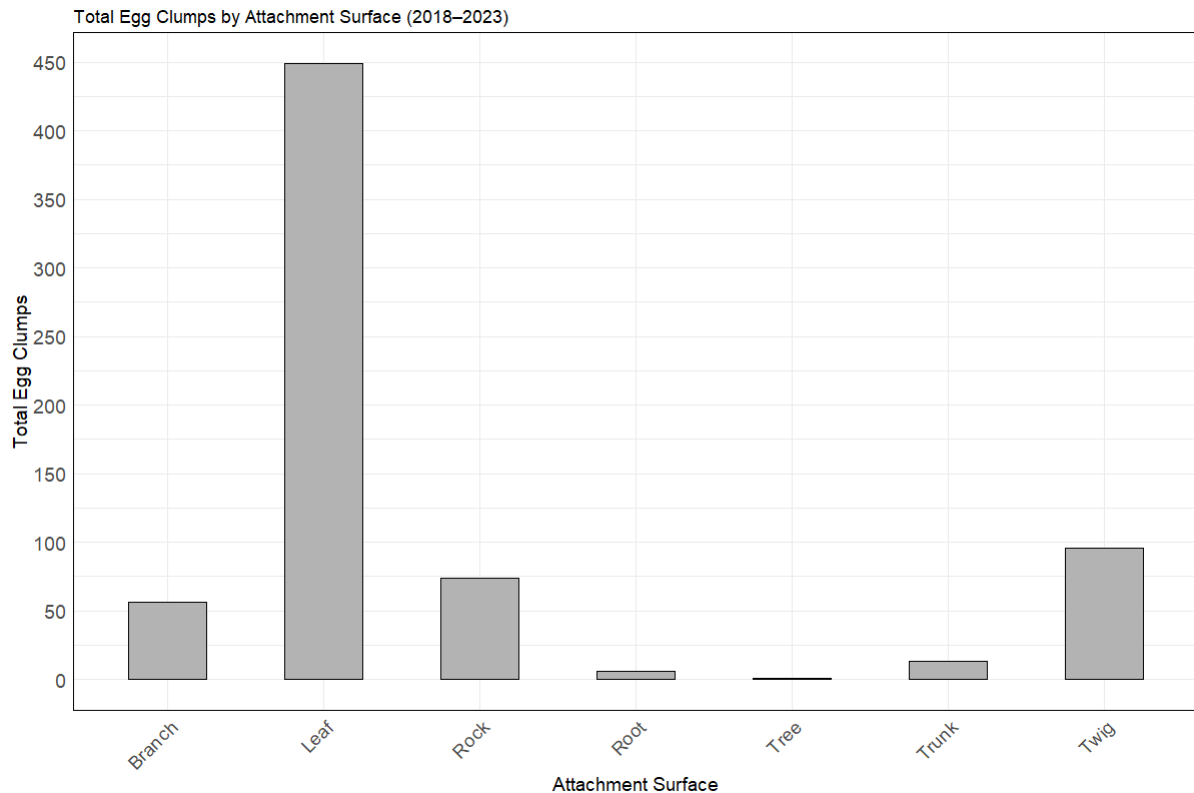


Figure 6. Bar graph of total egg clumps per attachment surface for the 2018–2023 timeframe.

The total number of egg clumps were displayed in binned height categories of 10 cm height intervals over the entire dataset (Figure 7), illustrating the height distribution of egg clumps. The mean height of recorded egg clumps over the entire dataset was 73.93 cm (SD = 43.94 cm). The mean height of recorded egg clumps over the 2013–2017 timeframe was 67.2 cm (SD = 40 cm), and 77.67 cm (SD = 47.2 cm) over the 2018–2023 timeframe – indicating a slight increase in height at which egg clumps were recorded between these two timeframes.

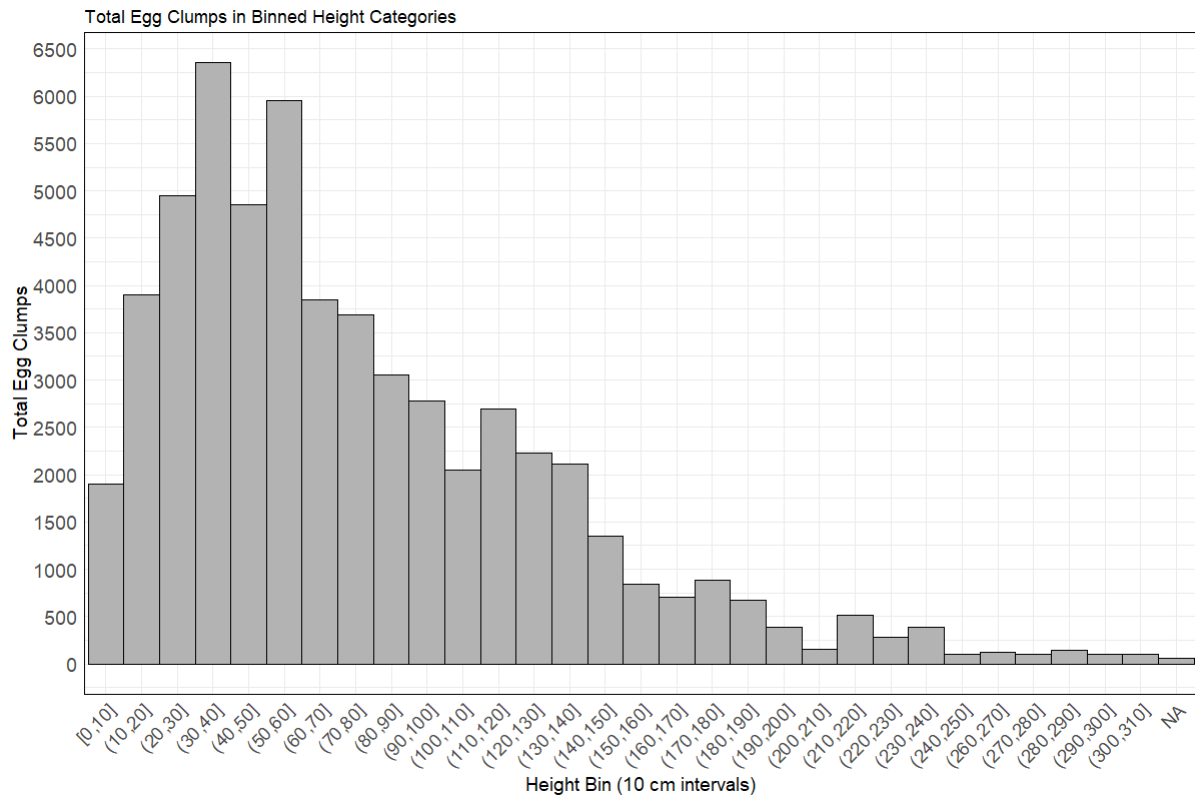


Figure 7. Bar graph of total egg clumps binned in height categories of 10 cm intervals for the entire dataset (2013–2023).

Boxplots were constructed to represent the mean monthly height of egg clumps for the 2013–2017 timeframe (Figure 8) and the 2018–2023 timeframe (Figure 9). Boxes were replaced with dots and whiskers were added to better illustrate the mean heights and spread of egg clumps. Multiple dots per month indicate the mean heights (cm) of recorded egg clumps for the different months in which data were recorded across both timeframes. Egg clumps recorded in April (2013–2017) were found at greater heights compared with other months. This was also evident for March and April of the 2018–2023 timeframe.

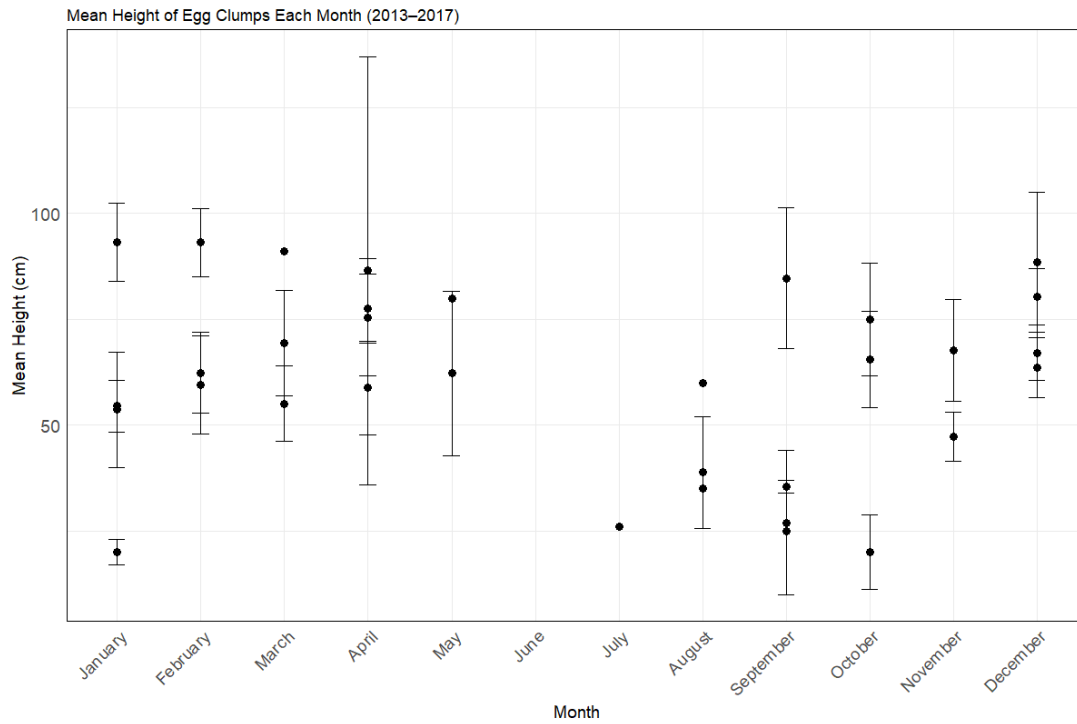


Figure 8. Line plot of mean egg clump height for each month of the 2013–2017 timeframe.

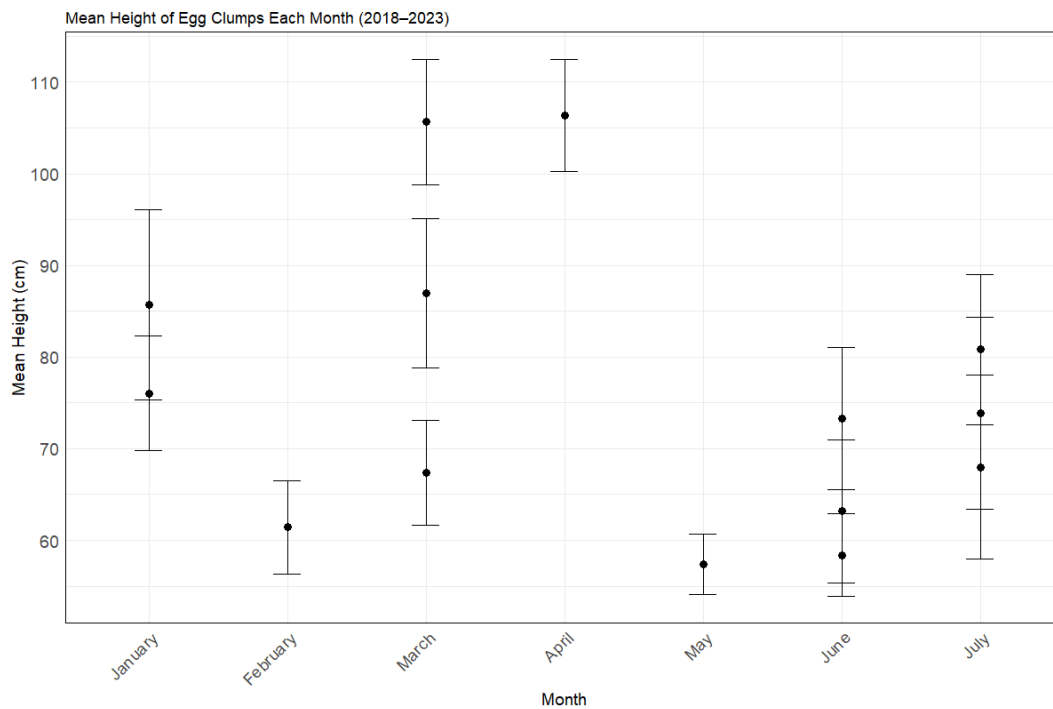


Figure 9. Line plot of mean egg clump height for each month of the 2018–2023 timeframe.

The maximum recorded height of egg clumps throughout 2013–2023 are illustrated in Figure 10. The highest recorded egg clumps were found in 2019 (288 cm), 2021 (265 cm) and 2023 (310 cm).

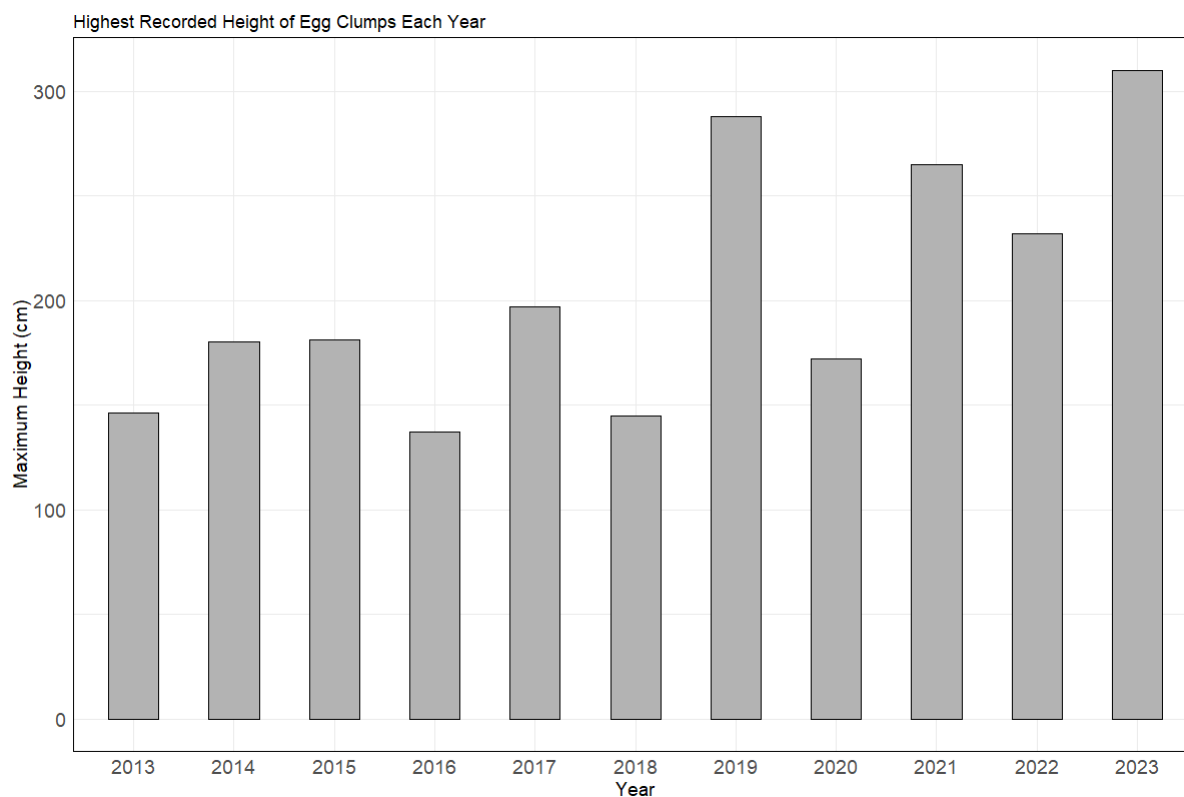


Figure 10. Bar graph of the highest recorded egg clumps each year from 2013 to 2023.

The number of eggs recorded in egg clumps across both timeframes are represented in dual-axis bar graphs in Figure 11. Monthly rainfall (mm) is overlayed with the number of eggs across the entire dataset (2013–2023).

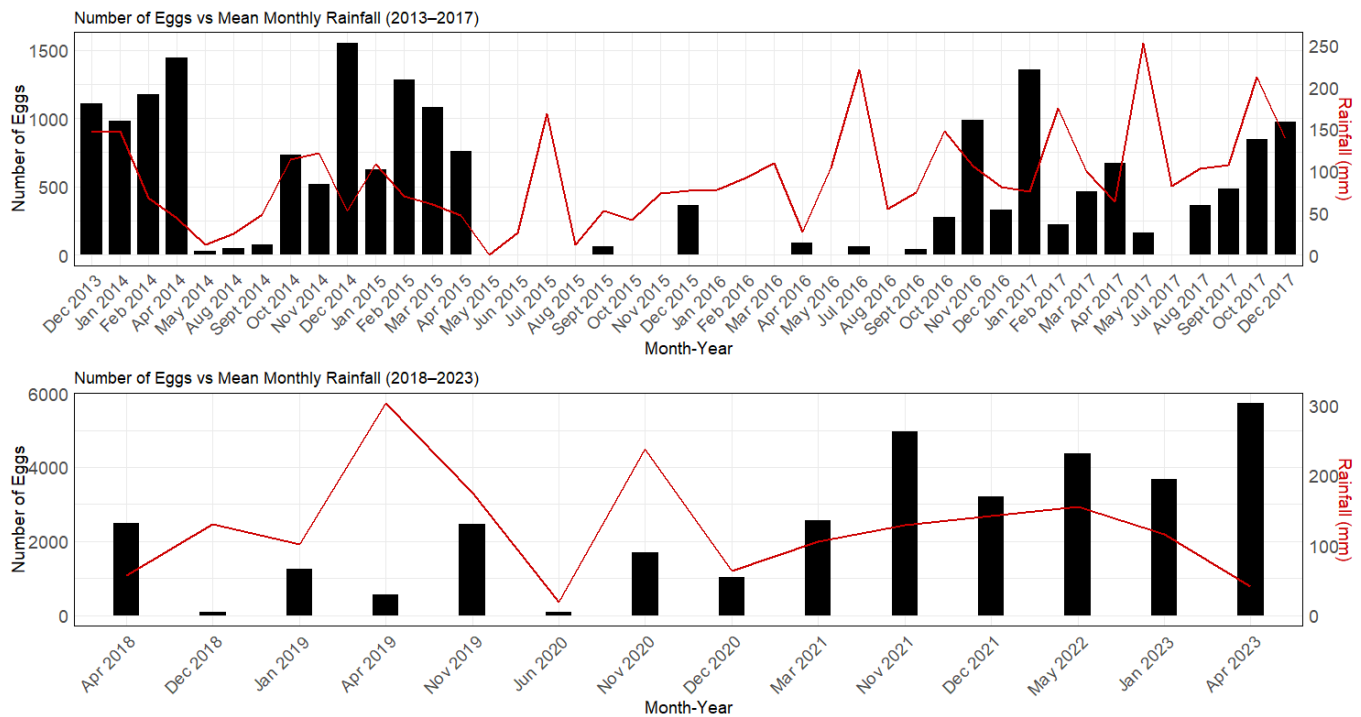


Figure 11. Dual-axis bar graphs depicting monthly rainfall overlayed with the number of eggs recorded in the 2013–2017 and 2018–2023 timeframes respectively. Number of eggs are depicted as black bars and monthly rainfall is depicted as a red line.

A boxplot was constructed to illustrate the relationship between monthly rainfall (mm) against development stage (Figure 12). Although all developmental stages are relatively evenly spread over a wide range of monthly rainfall values, the two developmental stages which characterise dried and dead egg clumps ('Drying' and 'Mouldy') were evident at much lower monthly rainfall values.

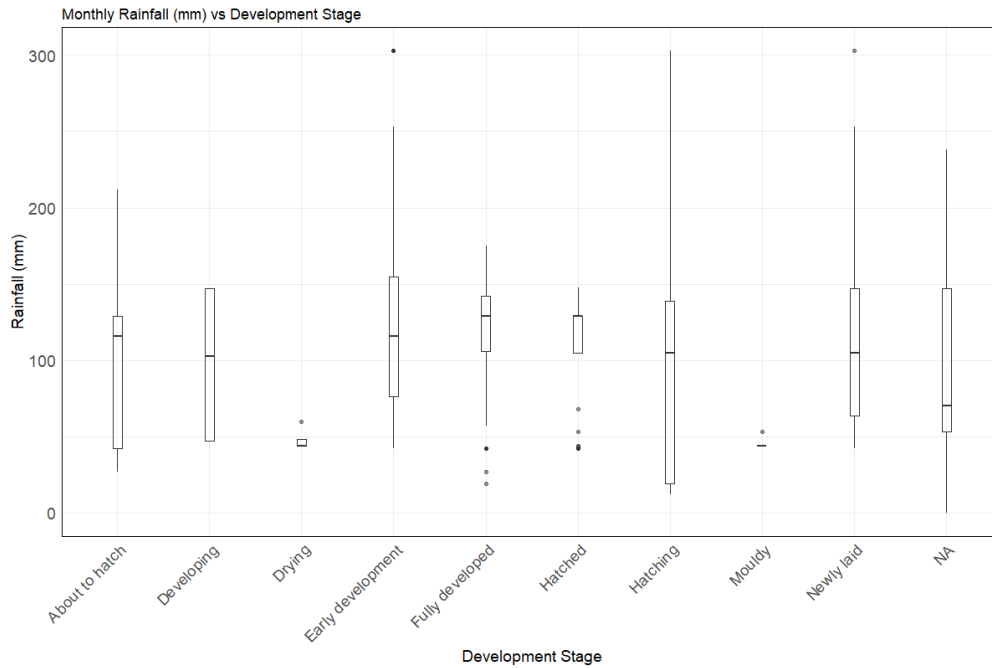


Figure 12. Boxplot of monthly rainfall (mm) vs the stage of development over the entire dataset (2013–2023).

A scatterplot was constructed (Figure 13) to illustrate the relationship between monthly rainfall (mm) and the heights (cm) at which egg clumps were recorded over the entire dataset. This scatterplot was fitted with a regression line and indicates a non-significant relationship between monthly rainfall and height of egg clumps ($R^2 < 0.001$).

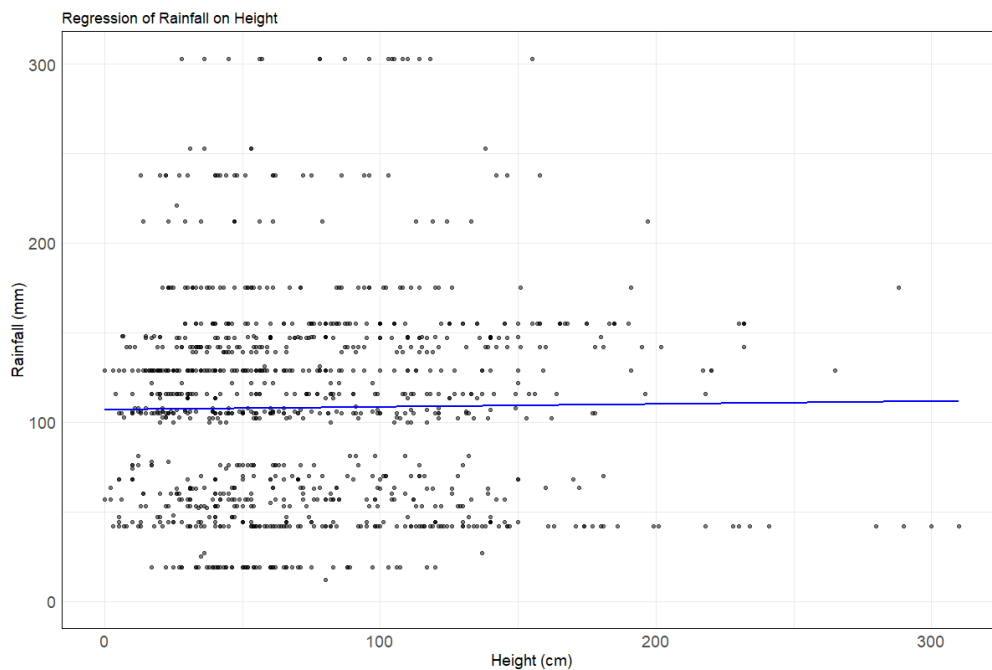


Figure 13. Scatterplot of monthly rainfall (mm) regressed with height of egg clumps over the entire dataset (2013–2023).

A similar scatterplot (Figure 14) was constructed to visualise the relationship between monthly rainfall (mm) and the number of egg clumps recorded for each month over the entire span of the data. A fitted regression line indicates a non-significant relationship between these two variables ($R^2 = 0.004$).

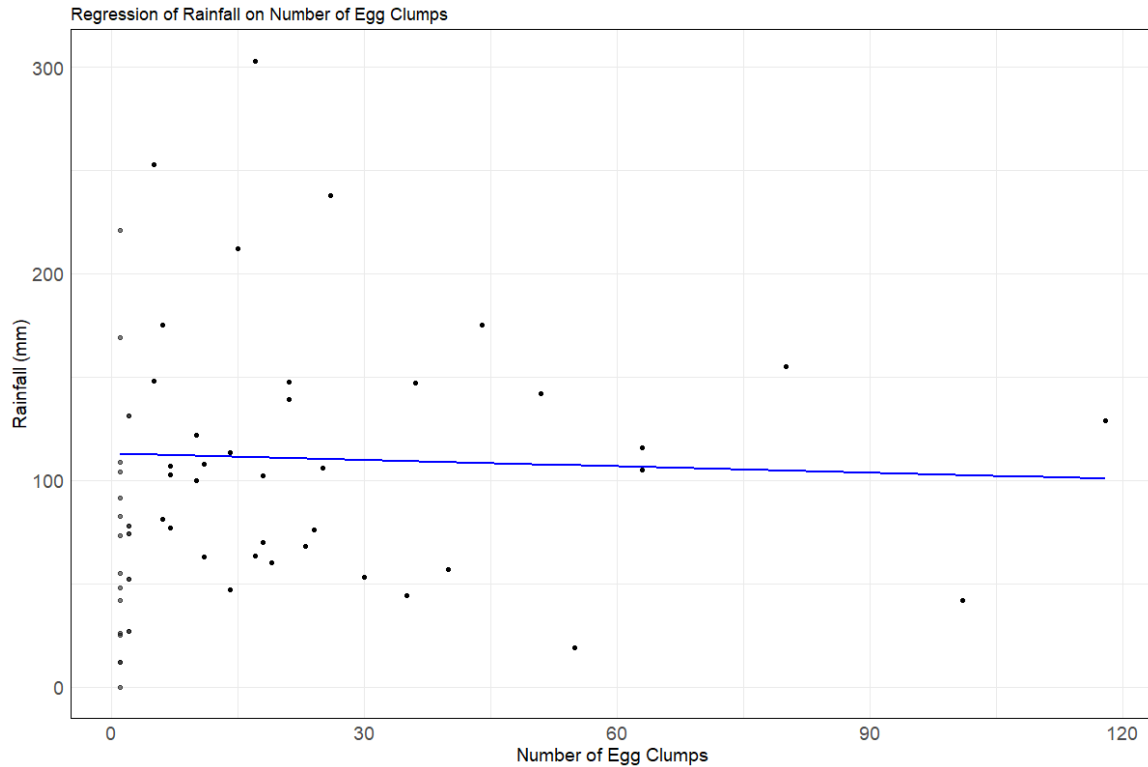


Figure 14. Scatterplot of monthly rainfall (mm) regressed over the number of egg clumps recorded over the entire dataset (2013–2023).

Another scatterplot was constructed (Figure 15) to illustrate the relationship between monthly rainfall (mm) and the number of eggs recorded in egg clumps. A fitted regression line also shows a slight negative relationship between an increase in monthly rainfall and number of eggs.

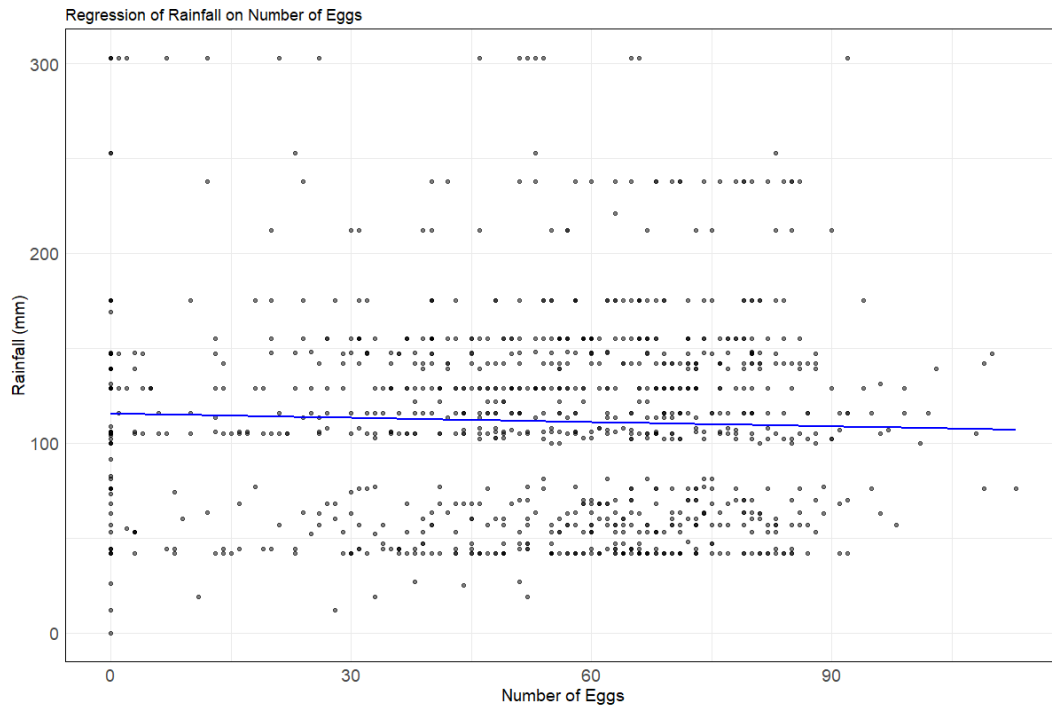


Figure 15. Scatterplot of monthly rainfall (mm) regressed over the number of eggs recorded over the entire dataset (2013–2023).

Monthly rainfall (mm) was also plotted against water depth recorded at egg clumps in a scatterplot (Figure 16). A fitted regression line indicates that an increase in water depth recorded at egg clumps was strongly associated with decreased recorded monthly rainfall.

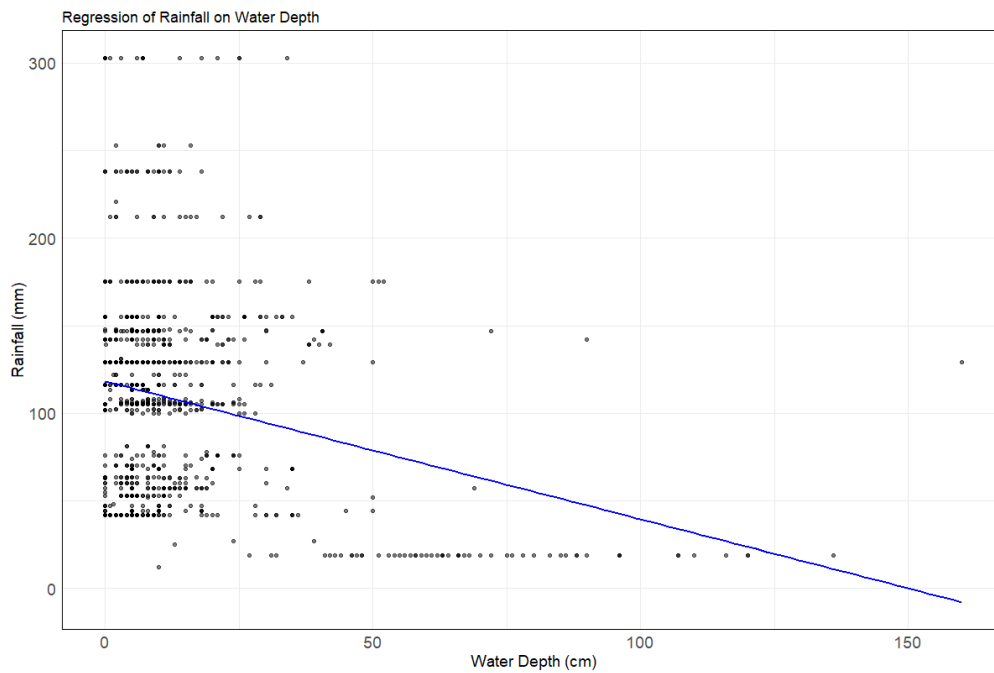


Figure 16. Scatterplot of monthly rainfall (mm) regressed over measured water depth (cm) at sites where egg clumps were recorded over the entire dataset (2013–2023).

Tile plots were constructed to illustrate the distribution of attachment surface (Figure 17) over months of the year for the entire dataset (2013–2023). The ‘rock’ attachment surface was recorded to be utilised each month year, while the ‘leaf’ and ‘twig’ attachment surfaces were also recorded to be used every month, except in July, which is outside of the *N. bonebergi* breeding season.

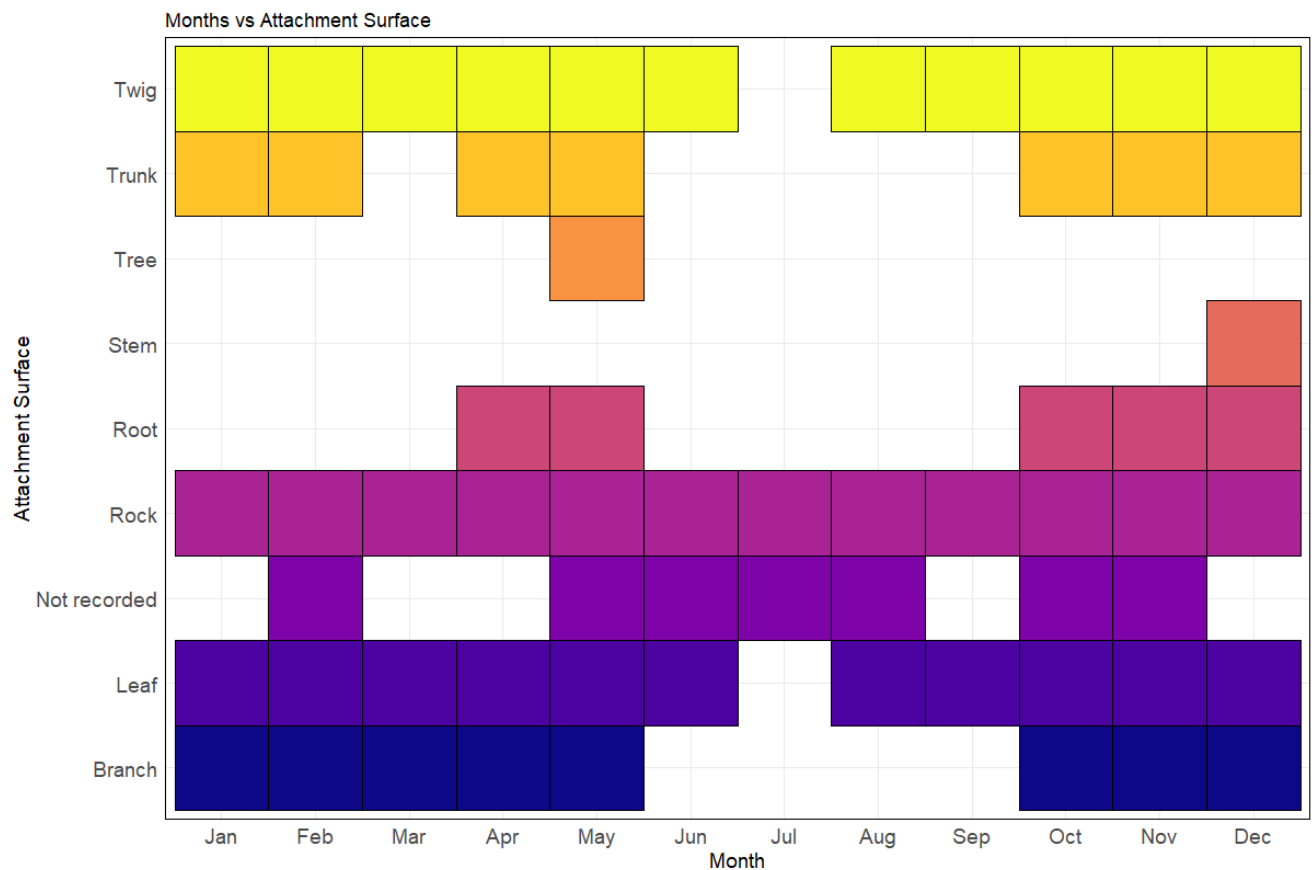


Figure 17. Tile plot of attachment surface distributed over every month in which egg clumps were recorded over the entire dataset (2013–2023).

A similar tile plot of the entire dataset illustrates developmental stages recorded each month (Figure 18). Early developmental stages ('newly laid', 'early development', 'developing') were not evident after the *N. bonebergi* breeding season concludes (June), however some advanced developmental stages ('fully developed', 'hatching') were recorded after the breeding season concluded. Some egg clumps in early development were recorded from July onwards – well before the start of the breeding season (October).

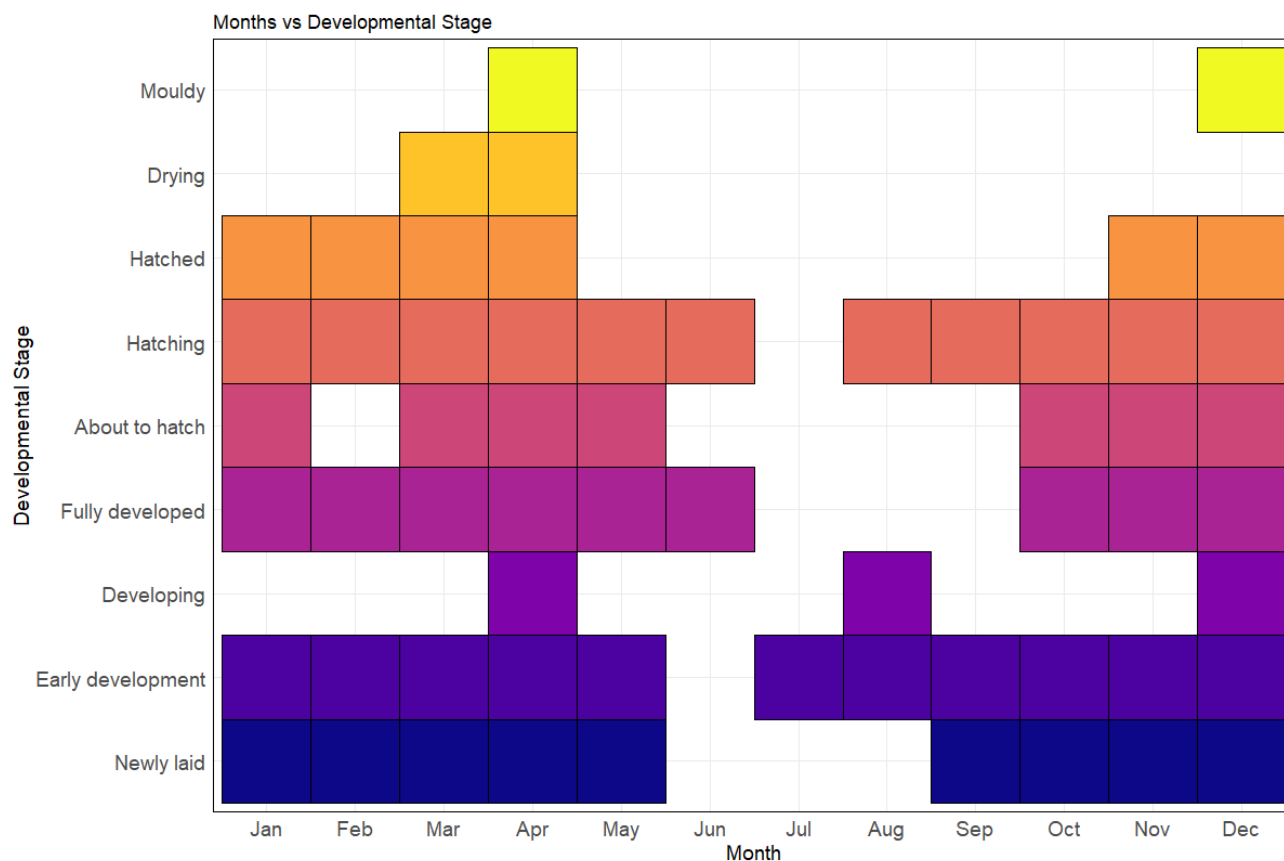


Figure 18. Tile plot of developmental stage of egg clumps distributed over every month in which egg clumps were recorded over the entire dataset (2013–2023).

Level of shade per month each year from 2013–2023 is illustrated in a tile plot (Figure 19). During the less active breeding season (May – October), fewer egg clumps were recorded in areas without full shade coverage ('full sun', 'light shade', 'partial shade'). However, egg clumps were recorded in areas with 'full shade' within and outside of the peak breeding seasons.

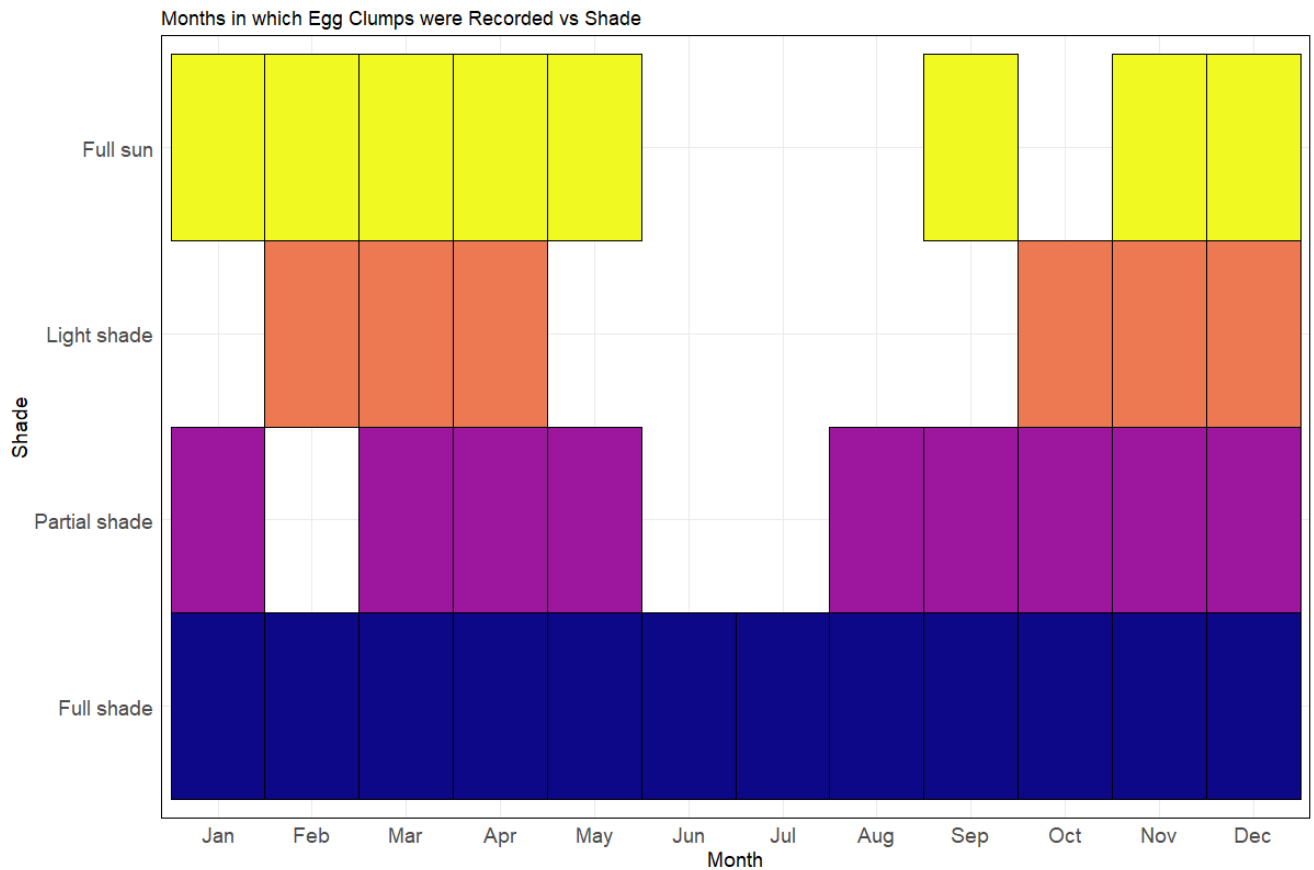


Figure 19. Tile plot of shade level recorded over every month of the entire dataset (2013–2023).

Summary statistics for the continuous variables measured from 2013–2023 are summarised in Table 1. Standard deviations of the number of egg clumps could not be obtained for the years 2013 and 2020 as egg clumps were only recorded in one month for each of these years.

Table 1. Summary statistics for egg clump monitoring data collected from 2013–2023 in the VCNR. Summary statistics are provided with means and standard deviations (sd) where applicable. Variables include year, total number of egg clumps, mean number of egg clumps, total number of eggs, mean number of eggs, mean height of egg clumps (cm), water depth at recorded egg clumps (cm), total rainfall (mm), and mean rainfall (mm).

Year	Total number of egg clumps	Number of egg clumps (mean $\pm$ sd)	Total number of eggs	Number of eggs (mean $\pm$ sd)	Height (cm) (mean $\pm$ sd)	Water depth (cm) (mean $\pm$ sd)	Total rainfall (mm)	Rainfall (mm) (mean $\pm$ sd)
2013	36	36	1110	44.4 $\pm$ 33.1	80.4 $\pm$ 40.0	12.7 $\pm$ 15.5	147	147
2014	136	25.0 $\pm$ 8.8	6552	48.2 $\pm$ 22.8	71.7 $\pm$ 43.9	9.9 $\pm$ 8.4	633	78.6 $\pm$ 39.2
2015	77	13.8 $\pm$ 5.7	4174	54.2 $\pm$ 23.6	65.7 $\pm$ 41.7	8.6 $\pm$ 8.4	736	67.7 $\pm$ 24.8
2016	47	15.0 $\pm$ 10.9	1790	39.8 $\pm$ 27.3	50.1 $\pm$ 36.1	10.4 $\pm$ 7.6	1094.5	102.4 $\pm$ 31.0
2017	107	15.2 $\pm$ 7.0	5547	51.8 $\pm$ 33.0	65.1 $\pm$ 38.4	13.8 $\pm$ 9.3	1310	126.0 $\pm$ 55.7
2018	42	38.2 $\pm$ 8.2	2575	61.3 $\pm$ 22.6	67.4 $\pm$ 35.5	13.2 $\pm$ 10.6	188	60.5 $\pm$ 16.0
2019	79	32.3 $\pm$ 13.3	4267	54.0 $\pm$ 26.2	79.1 $\pm$ 46.5	10.8 $\pm$ 11.5	580	185.9 $\pm$ 68.4
2020	98	40.7 $\pm$ 16.5	2832	61.6 $\pm$ 21.0	61.8 $\pm$ 33.0	42.0 $\pm$ 36.7	320.5	84.8 $\pm$ 93.9
2021	232	88.3 $\pm$ 30.5	10733	46.3 $\pm$ 28.0	64.2 $\pm$ 49.9	9.8 $\pm$ 14.0	376	125.3 $\pm$ 13.5

2022	80	80	4366	54.6 ± 16.9	106.4 ± 54.8	11.0 ± 11.0	155	155
2023	164	86.4 ± 18.5	9408	57.4 ± 22.0	94.3 ± 63.6	6.5 ± 8.6	158	70.4 ± 36.1

Linear models

Monthly rainfall had a highly significant effect ($p < 0.001$) on the number of egg clumps in the 2013–2017 timeframe. A coefficient estimate of $\beta = -0.075$ indicates a significant negative relationship between an increase in rainfall on the total number of egg clumps, holding all other variables constant in the model (height, water depth and shade). Partial shade was the only level of shade found to have a significant positive effect on the number of egg clumps over the 2013–2017 period ($\beta = 3.208$, $p = 0.009$) when all other variables were held constant in the linear model (rainfall, height and water depth). The same linear model was used for the egg clump monitoring data of the 2018–2023 timeframe. Both monthly rainfall ($\beta = -0.163$, $p < 0.001$) and water depth ($\beta = -0.363$, $p < 0.001$) were found to have significant negative effects on the number of egg clumps recorded.

The effect of height above water was significant ($p = 0.003$) and positive ($\beta = 0.115$) on the number of eggs per egg clump over the 2013–2017 timeframe, with all other model variables (rainfall, water depth and shade) being held constant. Similar results were maintained when this linear model was applied to the 2018–2023 egg clump monitoring data timeframe, where height above water was found to have a significant positive effect on the number of eggs in egg clumps ($\beta = 0.064$, $p < 0.001$). In this model, water depths at egg clump sites were found to have a significant effect ($p = 0.012$) that is negative ($\beta = -0.195$) on the number of eggs in egg clumps.

For the linear model constructed to determine the effects of variables on height, the water depth measured at egg clump sites variable was the only variable found to have a significant negative effect on the height above water at which egg clumps were laid ($\beta = -0.752$, $p = 0.004$) in the 2013–2017 timeframe, while keeping all other variables (rainfall and shade) constant in the model. Similar results were evident when this same model was applied to data from the 2018–2023 timeframe, where water depth was found to also have a significant negative effect on the height at which egg clumps were recorded during this timeframe ($\beta = -0.290$, $p = 0.005$).

A linear model was constructed to determine whether the rainfall and shade predictor variables related to water depth measured at sites where egg clumps were recorded. For the

2013–2017 timeframe, no significant relationship between variables was established, but for the 2018–2023 timeframe, rainfall had a highly significant ($p < 0.001$) and negative ($\beta = -0.116$) effect on the water depth variable. Partial shade had a highly significant ($p < 0.001$) and highly negative ($\beta = -6.852$) effect on the water depth variable for the 2018–2023 timeframe.

Multinomial regression models

Multinomial regression models were constructed for the categorical variables (attachment surface, attachment detail, level of shade and development stage), as these models yielded more accurate estimates of the categorical variables with numerous levels than regular linear models. Multinomial regression model outputs for the 2013–2017 and 2018–2023 timeframes are compiled in Tables 2–9. When modeling monthly rainfall against the developmental stage of recorded egg clumps in the 2013–2017 timeframe (Table 2), significant relationships were found for the ‘drying’ and ‘mouldy’ stages of egg clump development ($p = 0.021$ and $p = 0.01$, respectively). These significant associations to monthly rainfall were accompanied by slightly negative model coefficients for the ‘drying’ ($\beta = -0.072$) and ‘mouldy’ ($\beta = -0.079$) developmental stages. Indicating that increased rainfall may slightly decrease the number of drying and mouldy egg clumps found.

Table 2. Multinomial regression model outputs for monthly rainfall vs developmental stage (2013–2017). Coefficient estimate, standard error, p-value, lower confidence interval and upper confidence interval are presented for each developmental stage.

Predictor	Coefficient	Std error	p-value	CI lower	CI upper
Developing	–0.003	0.009	0.781	–0.021	0.016
Drying	–0.072	0.031	0.021	–0.133	–0.011
Early development	0.007	0.007	0.300	–0.007	0.022
Fully developed	0.003	0.009	0.707	–0.014	0.020
Hatched	0.001	0.010	0.932	–0.019	0.020
Hatching	0.004	0.007	0.596	–0.010	0.018
Mouldy	–0.079	0.030	0.010	–0.138	–0.019
Newly laid	0.007	0.007	0.327	–0.007	0.022

These two types of egg clumps were not recorded in the 2018–2023 timeframes. For this timeframe (Table 3), multinomial regression modeling of monthly rainfall against developmental stage of egg clumps yielded significant associations with the ‘early development’ and ‘fully developed’ developmental stages ($p = 0.039$ and $p = 0.007$,

respectively). However, model coefficients obtained for these two developmental stages indicate only slight positive relationship between monthly rainfall and developmental stage ($\beta = 0.006$ and $\beta = 0.009$, respectively). The ‘early development’ stage confidence intervals included zero (CI = [0, 0.012]), which indicate that the effect of monthly rainfall on this variable might be weak.

Table 3. Multinomial regression model output for monthly rainfall vs developmental stage (2018–2023). Coefficient estimate, standard error, p-value, lower confidence interval and upper confidence interval are presented for each developmental stage.

Predictor	Coefficient	Std error	p-value	CI lower	CI upper
Early development	0.006	0.003	0.039	0.000	0.012
Fully developed	0.009	0.003	0.007	0.002	0.015
Hatched	0.005	0.004	0.143	−0.002	0.012
Hatching	−0.002	0.003	0.571	−0.008	0.004
Newly laid	0.003	0.003	0.452	−0.004	0.009

Multinomial regression models constructed for monthly rainfall and attachment surface for the 2013–2017 timeframe (Table 4) indicated that the ‘leaf’ and ‘rock’ attachment surfaces were significantly associated with monthly rainfall ($p = 0.019$ and $p = 0.044$, respectively). These two attachment surfaces had slight positive relationships with monthly rainfall ($\beta = 0.009$ and 0.011 , respectively), however, since the confidence interval ranges for both of these variables included or very nearly included zero (CI = [0.001, 0.017]; CI = [0, 0.021], respectively), the relationship between rainfall and these two predictors might be weak. No significant relationships were found between monthly rainfall and attachment surface for the 2018–2023 timeframe (Table 5).

Table 4. Multinomial regression model output for monthly rainfall vs attachment surface (2013–2017). Coefficient estimate, standard error, p-value, lower confidence interval and upper confidence interval are presented for each attachment surface.

Predictor	Coefficient	Std error	p-value	CI lower	CI upper
Leaf	0.009	0.004	0.019	0.001	0.017
Not recorded	−0.014	0.012	0.221	−0.037	0.008
Rock	0.011	0.005	0.044	0.000	0.021
Root	0.012	0.010	0.254	−0.009	0.032
Stem	−0.035	0.043	0.420	−0.118	0.049

Trunk	−0.005	0.010	0.637	−0.024	0.014
Twig	0.003	0.005	0.569	−0.007	0.013

Table 5. Multinomial regression model output for monthly rainfall vs attachment surface (2018–2023). Coefficient estimate, standard error, p-value, lower confidence interval and upper confidence interval are presented for each attachment surface.

Predictor	Coefficient	Std error	p-value	CI lower	CI upper
Leaf	−0.003	0.002	0.175	−0.008	0.001
Rock	−0.004	0.003	0.198	−0.009	0.002
Root	0.007	0.006	0.280	−0.005	0.018
Tree	0.007	0.014	0.593	−0.020	0.034
Trunk	0.006	0.004	0.206	−0.003	0.014
Twig	0.000	0.003	0.963	−0.005	0.005

Multinomial regression outputs for monthly rainfall modeled with attachment detail are laid out in Table 6 for the 2013–2017 timeframe and Table 7 for the 2018–2023 timeframe. Numerous plant taxa were significantly associated with monthly rainfall, and for the 2013–2017 timeframe these significant relationships were positively connected to monthly rainfall. However, all significant relationships of attachment details associated with monthly rainfall in the 2018–2023 timeframe were negative, save for *Septio cordea*, which was the only significantly positive relationship ($\beta = 0.1$, $p = < 0.001$).

Table 6. Multinomial regression model output for monthly rainfall vs attachment detail (2013–2017). Coefficient estimate, standard error, p-value, lower confidence interval and upper confidence interval are presented for each attachment detail.

Predictor	Coefficient	Std error	p-value	CI lower	CI upper
<i>Ageratina adenophora</i>	0.055	0.024	0.025	0.007	0.103
<i>Allophylus dregeanus</i>	0.045	0.019	0.015	0.009	0.082
<i>Baphia racemosa</i>	−0.009	0.023	0.705	−0.054	0.036
<i>Cassipourea gummiflua</i>	0.062	0.020	0.002	0.022	0.102
<i>Chromolaena stem</i>	−0.037	0.051	0.478	−0.137	0.064
Climbing bamboo	0.012	0.038	0.754	−0.063	0.087
<i>Cyathia dregei</i>	0.048	0.021	0.025	0.006	0.090
<i>Cyperus dives</i>	−0.014	0.032	0.669	−0.076	0.049
<i>Dalbergia armata</i>	0.041	0.027	0.134	−0.012	0.094
Dead branch	0.032	0.019	0.085	−0.004	0.069
Dead tree	0.035	0.023	0.129	−0.010	0.081
Dead twig	0.040	0.022	0.077	−0.004	0.084

<i>Dryopteris</i> spp.	−0.015	0.050	0.773	−0.113	0.084
<i>Englerophytum natalense</i>	0.029	0.016	0.077	−0.003	0.061
Fallen tree	0.020	0.027	0.463	−0.033	0.072
Fern	0.041	0.017	0.012	0.009	0.074
<i>Ficus sur</i>	0.048	0.021	0.025	0.006	0.090
<i>Grewia lasiocarpa</i>	0.014	0.037	0.697	−0.058	0.087
<i>Hedychium gardnerianum</i>	0.053	0.017	0.002	0.019	0.087
<i>Impatiens hochstetteri</i>	−0.005	0.026	0.858	−0.055	0.045
<i>Kniphofia gracilis</i>	0.000	0.045	0.999	−0.087	0.087
<i>Kniphofia</i> spp.	0.012	0.038	0.753	−0.063	0.087
<i>Maesa lanceolata</i>	0.033	0.017	0.061	−0.002	0.067
<i>Monanthes affinis</i>	0.048	0.026	0.060	−0.002	0.098
<i>Oplismenus hirtellus</i>	0.058	0.024	0.016	0.011	0.105
<i>Phoenix reclinata</i>	0.057	0.021	0.005	0.017	0.098
<i>Phragmites</i>	0.000	0.045	0.999	−0.088	0.087
<i>Plectranthus</i> spp.	0.012	0.038	0.753	−0.063	0.087
<i>Plectranthus zuluensis</i>	0.000	0.045	0.999	−0.088	0.087
<i>Protorhus longifolia</i>	−0.015	0.038	0.697	−0.088	0.059
<i>Rhoicissus tomentosa</i>	0.055	0.024	0.025	0.007	0.103
Rock	0.041	0.017	0.015	0.008	0.073
Root	0.050	0.021	0.018	0.009	0.091
<i>Rubus rosifolius</i>	−0.015	0.050	0.772	−0.113	0.084
<i>Scadoxus multiflorus</i>	0.040	0.017	0.017	0.007	0.072
<i>Scadoxus</i> spp.	0.023	0.017	0.180	−0.011	0.056
Small leaf	0.058	0.017	0.001	0.025	0.090
Small tree	0.012	0.021	0.575	−0.030	0.054
<i>Smilax anceps</i>	0.023	0.023	0.319	−0.022	0.068
Stem	0.041	0.027	0.134	−0.012	0.094
<i>Strelitzia nicolai</i>	0.041	0.027	0.134	−0.012	0.094
Unidentified	0.051	0.016	0.002	0.019	0.083
<i>Zantedeschia aethiopica</i>	0.039	0.022	0.081	−0.005	0.083
<i>Zantedeschia albomaculata</i>	−0.015	0.050	0.772	−0.113	0.084

Table 7. Multinomial regression model output for monthly rainfall vs attachment detail (2018–2023). Coefficient estimate, standard error, p-value, lower confidence interval and upper confidence interval are presented for each attachment detail.

Predictor	Coefficient	Std error	p-value	CI lower	CI upper
<i>Allophylus dregeanus</i>	−0.038	0.005	< 0.001	−0.048	−0.027
<i>Allophylus natalensis</i>	−0.015	0.009	0.108	−0.034	0.003

<i>Apodytes dimidiata</i>	−0.039	0.007	< 0.001	−0.052	−0.026
<i>Asparagus falcatus</i>	−0.025	0.009	0.007	−0.043	−0.007
<i>Asparagus</i> spp.	−0.015	0.015	0.320	−0.045	0.015
<i>Baphia racemosa</i>	−0.033	0.006	< 0.001	−0.046	−0.020
<i>Bridelia micrantha</i>	−0.022	0.007	0.001	−0.035	−0.009
<i>Bridelia</i> spp.	−0.056	0.011	< 0.001	−0.079	−0.034
Broad leaf bristle grass	−0.277	0.171	0.104	−0.612	0.057
<i>Croton sylvaticus</i>	−0.015	0.015	0.318	−0.045	0.015
<i>Cyperus</i> spp.	−0.003	0.015	0.855	−0.032	0.026
<i>Dalbergia armata</i>	−0.029	0.006	< 0.001	−0.041	−0.017
<i>Dalbergia</i> spp.	−0.039	0.021	0.061	−0.080	0.002
Dead branch	−0.024	0.019	0.196	−0.061	0.012
Dead twig	−0.058	0.024	0.016	−0.106	−0.011
<i>Englerophytum natalense</i>	−0.029	0.005	< 0.001	−0.038	−0.020
Fern	−0.061	0.009	< 0.001	−0.079	−0.043
<i>Ficus burtt-davyi</i>	−0.020	0.010	0.053	−0.041	0.000
<i>Ficus sur</i>	−0.028	0.012	0.018	−0.052	−0.005
<i>Grewia lasiocarpa</i>	−0.021	0.009	0.014	−0.038	−0.004
<i>Impatiens hochstetteri</i>	−0.034	0.021	0.098	−0.075	0.006
<i>Impatiens</i> spp.	−0.048	0.011	< 0.001	−0.071	−0.026
<i>Iris</i> spp.	−0.034	0.021	0.098	−0.075	0.006
<i>Maesa lanceolata</i>	−0.031	0.010	0.002	−0.050	−0.012
<i>Maesa</i> spp.	−0.039	0.021	0.061	−0.080	0.002
<i>Monanthotaxis caffra</i>	−0.055	0.005	< 0.001	−0.065	−0.044
<i>Phoenix reclinata</i>	−0.036	0.007	< 0.001	−0.050	−0.021
<i>Plectranthus</i> spp.	−0.062	0.026	0.018	−0.114	−0.011
<i>Protorhus longifolia</i>	−0.020	0.007	0.005	−0.034	−0.006
<i>Pteridium aquilinum</i>	−0.022	0.013	0.086	−0.048	0.003
<i>Rhoicissus tomentosa</i>	−0.015	0.011	0.175	−0.037	0.007
Rock	−0.038	0.005	< 0.001	−0.048	−0.028
Root	−0.024	0.014	0.072	−0.051	0.002
<i>Scadoxus multiflorus</i>	−0.017	0.005	< 0.001	−0.026	−0.008
<i>Scadoxus</i> spp.	−0.064	0.006	< 0.001	−0.076	−0.052
<i>Septio cordea</i>	0.100	0.004	< 0.001	0.091	0.109
<i>Seteria</i> spp.	−0.062	0.026	0.018	−0.114	−0.011
<i>Strelitzia nicolai</i>	−0.020	0.009	0.029	−0.038	−0.002
<i>Syzygium cordatum</i>	−0.033	0.015	0.026	−0.062	−0.004
Unidentified	−0.037	0.005	< 0.001	−0.047	−0.028

Vine	−0.240	0.272	0.377	−0.773	0.293
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Multinomial regression models were constructed for investigating the relationships between monthly rainfall (mm) and level of shade for both timeframes. ‘Full shade’ was absent from model outputs for both timeframes, as this variable was used as the reference category for these models. This was done because the ‘full shade’ variable had the most recorded data entries in each timeframe, which increased the stability and reduced standard errors of both models. ‘Full shade’ therefore served as the baseline by which the other shade categories were compared with. In the multinomial regression model constructed for the 2013–2017 timeframe (Table 8) all three categories of shade were negative and significantly associated with monthly rainfall: full sun ($\beta = -0.02$, $p = 0.010$); light shade ($\beta = -0.017$, $p = 0.001$); partial shade ($\beta = -0.011$, $p = 0.001$). When the same model was used for the 2018–2023 timeframe (Table 9), only the ‘full sun’ level of shade was significantly associated with monthly rainfall ($p < 0.001$) and the relationship was positive ($\beta = 0.019$). Partial shade did not have a significant relationship with monthly rainfall for this timeframe.

Table 8. Multinomial regression model output for monthly rainfall vs level of shade (2013–2017). Coefficient estimate, standard error, p-value, lower confidence interval and upper confidence interval are presented for each level of shade.

Predictor	Coefficient	Std error	p-value	CI lower	CI upper
Full sun	−0.020	0.008	0.010	−0.036	−0.005
Light shade	−0.017	0.005	0.001	−0.027	−0.007
Partial shade	−0.011	0.003	0.001	−0.017	−0.004

Table 9. Multinomial regression model output for monthly rainfall vs level of shade (2018–2023). Coefficient estimate, standard error, p-value, lower confidence interval and upper confidence interval are presented for each level of shade.

Predictor	Coefficient	Std error	p-value	CI lower	CI upper
Full sun	0.019	0.004	< 0.001	0.011	0.028
Partial shade	−0.001	0.001	0.375	−0.004	0.002

3.1.2 Egg clump isolation experiments

Experimental designs for the egg clump isolation experiments (ECIE) carried out in February and April of 2024 are laid out in Tables 10 and 11, respectively. These tables indicate the attachment surface, height above water level (cm), setup date and time, end date and time and the level of shade at oviposition sites where egg clump development was recorded.

Table 10. Egg clump isolation experiment: February 2024. ECI = Egg clump isolation treatment, Control = Control treatment.

Egg clump ID	Attachment Surface	Height (cm)	Setup date	End date	Setup time	End time	Shade
ECI_1	Branch	106	02-Feb	05-Feb	18:50	12:10	Light shade
ECI_2	Leaf	45	02-Feb	04-Feb	19:00	10:30	Light shade
ECI_3	Leaf	94	02-Feb	09-Feb	21:15	19:30	Full shade
ECI_4	Leaf	140	03-Feb	07-Feb	10:10	10:45	Partial shade
ECI_5	Twig	80	03-Feb	09-Feb	11:20	19:30	Partial shade
ECI_6	Dead branch	8	03-Feb	09-Feb	11:55	19:45	Full shade
ECI_7	Leaf	142	03-Feb	09-Feb	12:00	19:55	Full shade
ECI_8	Twig	240	03-Feb	07-Feb	19:30	12:00	Partial shade
ECI_9	Leaf	20	05-Feb	09-Feb	10:50	17:10	Light shade
ECI_10	Leaf	243	05-Feb	09-Feb	11:40	17:30	Partial shade
ECI_11	Leaf	63	05-Feb	08-Feb	11:57	10:30	Full shade
ECI_12	Leaf	14	07-Feb	09-Feb	12:35	20:10	Full shade
Control_1	Leaf	65	05-Feb	07-Feb	09:30	12:10	Light shade

Control_2	Leaf	63	06-Feb	09-Feb	10:10	16:55	Light shade
Control_3	Grass	37	07-Feb	09-Feb	11:06	16:55	Partial shade
Control_4	Leaf	70	04-Feb	08-Feb	11:00	10:15	Partial shade
Control_5	Leaf	40	04-Feb	09-Feb	11:05	17:10	Full shade
Control_6	Leaf	105	02-Feb	09-Feb	21:15	19:30	Partial shade
Control_7	Leaf	140	04-Feb	09-Feb	11:35	17:45	Partial shade
Control_8	Leaf	160	08-Feb	09-Feb	11:25	17:45	Partial shade
Control_9	Leaf	153	08-Feb	09-Feb	11:25	17:45	Partial shade
Control_10	Leaf	153	09-Feb	09-Feb	10:35	17:45	Partial shade
Control_11	Leaf	142	08-Feb	09-Feb	12:05	19:55	Full shade
Control_12	Vine	254	03-Feb	07-Feb	19:30	12:00	Partial shade
Control_13	Twig	15	05-Feb	09-Feb	11:05	17:25	Light shade
Control_14	Leaf	235	05-Feb	06-Feb	12:35	11:30	Partial shade
Control_15	Vine	67	05-Feb	07-Feb	12:05	10:25	Full shade
Control_16	Leaf	127	06-Feb	09-Feb	11:35	17:05	Partial shade

Table 11. Egg clump isolation experiment: April 2024. ECM = Egg clump moistening treatment, ECI = Egg clump isolation treatment, Control = Control treatment.

Egg clump ID	Attachment surface	Height (cm)	Setup date	End date	Setup time	End time	Shade
ECM_1	Dead branch	22	14-Apr	22-Apr	09:00	08:52	Light shade
ECM_2	Blade	22	14-Apr	22-Apr	10:38	09:06	Full shade
ECM_3	Leaf	175	14-Apr	22-Apr	11:56	09:55	Partial shade
ECM_4	Leaf	168	15-Apr	22-Apr	10:57	10:14	Partial shade
ECM_5	Branch	63	15-Apr	22-Apr	11:25	10:21	Light shade
ECI_1	Leaf	52	14-Apr	22-Apr	10:45	09:10	Full shade
ECI_2	Leaf	46	14-Apr	22-Apr	10:49	09:19	Full shade
ECI_3	Leaf	144	14-Apr	22-Apr	11:30	09:40	Light shade
ECI_4	Leaf	154	14-Apr	22-Apr	15:12	10:06	Partial shade
ECI_5	Leaf	42	14-Apr	22-Apr	15:50	10:25	Full shade
ECI_6	Leaf	45	15-Apr	22-Apr	10:20	09:46	Partial shade
ECI_7	Leaf	235	16-Apr	22-Apr	21:07	10:31	Light shade
Control_1	Leaf	79	14-Apr	22-Apr	11:00	09:17	Full shade
Control_2	Leaf	89	14-Apr	22-Apr	10:55	09:23	Full shade
Control_3	Leaf	36	14-Apr	22-Apr	11:32	09:43	Partial shade
Control_4	Leaf	157	14-Apr	22-Apr	15:15	10:13	Light shade

Control_5	Leaf	54	14-Apr	22-Apr	15:50	10:17	Partial shade
Control_6	Leaf	83	15-Apr	22-Apr	10:25	09:53	Partial shade
Control_7	Leaf	217	16-Apr	22-Apr	21:16	10:35	Light shade

Summary statistics for the February 2024 ECIE egg clump isolation experiment (ECI) treatment are compiled in Table 12, and the control treatment is compiled in Table 13. The number of records, mean, standard deviation, median, minimum, maximum, range, skew, kurtosis and standard error are presented for each variable in both tables.

Table 12. Summary statistics for egg clump isolation experiment: Experimental treatment (February 2024).

Variable	n	mean	sd	median	min	max	range	skew	kurtosis	se
Gosner Stage	69	7.0	3.1	7	1	13	12	-0.1	-0.7	0.38
Temp	68	25.0	1.8	25.6	20.6	27.7	7.1	-1.0	0.1	0.22
RH	68	81.6	8.5	81.3	54.6	96.5	41.9	-0.4	0.2	1.03
Height	69	99.9	75.1	94.0	8.0	243.0	235.0	0.7	-0.5	9.04
Shade	69	1.9	0.9	2	1	3	2	0.1	-1.7	0.11
Substrate	69	2.0	0.6	2	1	3	2	-0.01	-0.3	0.07

Table 13. Summary statistics for egg clump isolation experiment: Control treatment (February 2024).

Variable	n	mean	sd	median	min	max	range	skew	kurtosis	se
Gosner Stage	69	12.4	5.0	13	1	21	20	-0.6	-0.3	0.6
Temp	53	24.7	2.1	25.6	20.6	28.2	7.6	-0.7	-0.6	0.3
RH	53	83.5	8.9	84.0	66.8	99.7	32.9	-0.2	-1.0	1.22
Height	69	107.6	68.8	105.0	15.0	254.0	239.0	0.7	-0.4	8.28
Shade	69	2.4	0.8	3	1	3	2	-0.9	-0.8	0.1
Substrate	69	2.3	0.8	2	1	4	3	1.2	0.7	0.09

The April ECIE summary statistics for the egg clump moistening (ECM) treatment are compiled in Table 14, the ECI treatment summary statistics are in Table 15 and the Control treatment summary statistics are compiled in Table 16. The number of records, mean, standard

deviation, median, minimum, maximum, range, skew, kurtosis and standard error are presented for each variable in all three tables.

Table 14. Summary statistics for egg clump isolation experiment: Egg clump moistening treatment (April 2024).

Variables	n	mean	sd	median	min	max	range	skew	kurtosis	se
Gosner Stage	40	9.2	4.3	9.5	1	17	16.	-0.31	-1.0	0.69
Temp	40	21.1	1.8	20.6	19.0	26.7	7.7	1.3	1.8	0.28
RH	40	93.9	7.6	96.8	76.5	102.2	25.7	-1.0	-0.4	1.20
Height	40	82.9	68.4	63	22	175	153	0.5	-1.7	10.82
Shade	40	2.1	0.8	2	1	3	2	-0.2	-1.3	0.12
Substrate	40	1.9	0.9	2	1	3	2	0.1	-1.8	0.14

Table 15. Summary statistics for egg clump isolation experiment: Egg clump isolation treatment (April 2024).

Variables	n	mean	sd	median	min	max	range	skew	kurtosis	se
Gosner Stage	57	10.2	4.8	10	1	20	19	-0.1	-0.8	0.63
Temp	57	20.8	1.6	20.6	18.6	28.1	9.5	1.8	5.2	0.22
RH	50	94.4	8.4	97.6	58.2	101.7	43.6	-2.5	6.7	1.19
Height	57	96.8	69.0	52	42	235.0	193	0.9	-0.7	9.13
Shade	57	1.8	0.9	2	1	3	2	0.3	-1.6	0.12
Substrate	57	1	0	1	1	1	0	NA	NA	0

Table 16. Summary statistics for egg clump isolation experiment: Control treatment (April 2024).

Variables	n	mean	sd	median	min	max	range	skew	kurtosis	se
Gosner Stage	59	8.8	4.6	8	1	19	18	0.4	-0.5	0.60
Temp	59	20.7	1.3	20.6	18.6	23.6	5.0	0.6	-0.5	0.16
RH	44	95.1	5.7	97.2	73.2	101.7	28.5	-2.0	4.1	0.85
Height	59	99.4	56.7	83	36	217	181	0.9	-0.3	7.38
Shade	59	2.1	0.9	2	1	3	2	-0.2	-1.6	0.11
Substrate	59	1	0	1	1	1	0	NA	NA	0

Time-series plots were constructed for both February 2024 ECIE treatments. The ECIE is illustrated in Figure 20, and shows how egg clump development (Gosner Stage) progressed over time. Development occurred rapidly initially, then started to level out after approximately 2 days of being isolated from female parental care. Only three of the twelve isolated egg clumps continued with development after the experiment concluded. Eight isolated egg clumps desiccated (indicated in Figure 20 as asterisks) and one was preyed upon by *Crematogaster* spp. ants (indicated in Figure 20 by a cross).

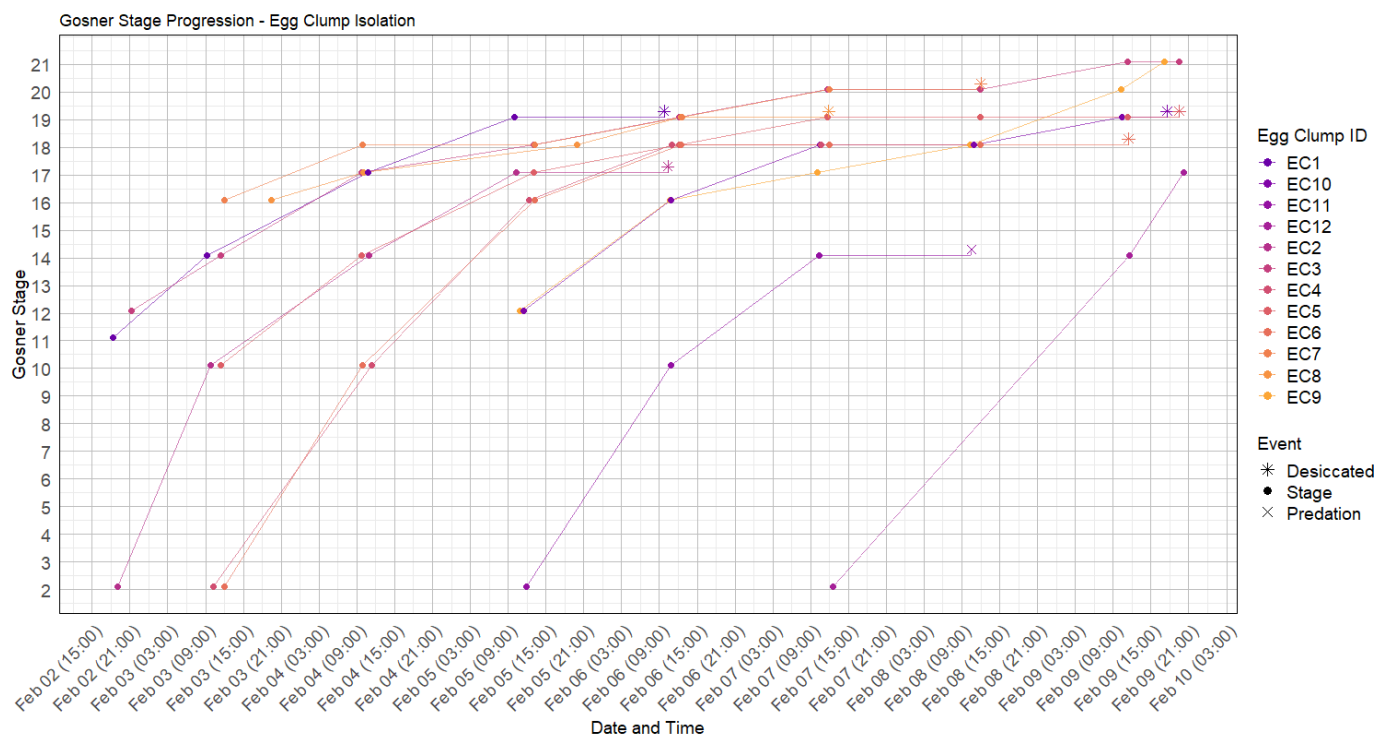


Figure 20. Time-series plot depicting Gosner Stage progression over time for the February 2024 egg clump isolation experiment. Desiccation and predation events are depicted with asterisks and a cross, respectively for the egg clump isolation treatment.

The Control of the February 2024 ECIE is shown in Figure 21. Three egg clumps hatched (illustrated by the plus symbol in Figure 21), and all others survived and continued with development after the ECIE concluded.

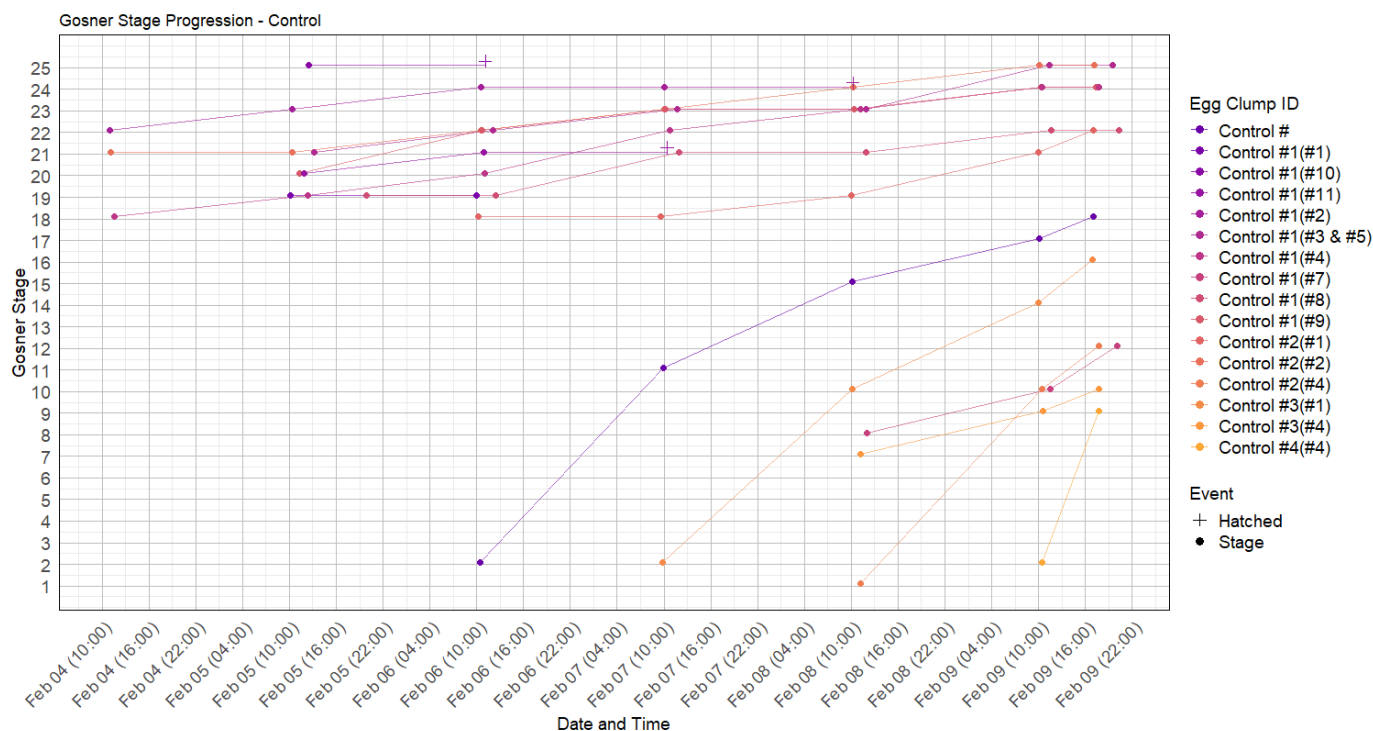


Figure 21. Time-series plot depicting Gosner Stage progression over time for the February 2024 egg clump isolation experiment. The control group depicts hatching events with a plus symbol.

For the April 2024 ECIE treatments, time-series plots were also constructed for the two treatments: egg clump moistening (ECM) and egg clump isolation (ECI) as well as the control group (Figures 22, 23 and 24, respectively) to illustrate the Gosner Stages of embryonic development over time for each group.

The time-series for the ECM treatment is shown in Figure 22, where isolated egg clumps were artificially moistened with a pipette every 24 hours. Only one of the five isolated egg clumps desiccated.

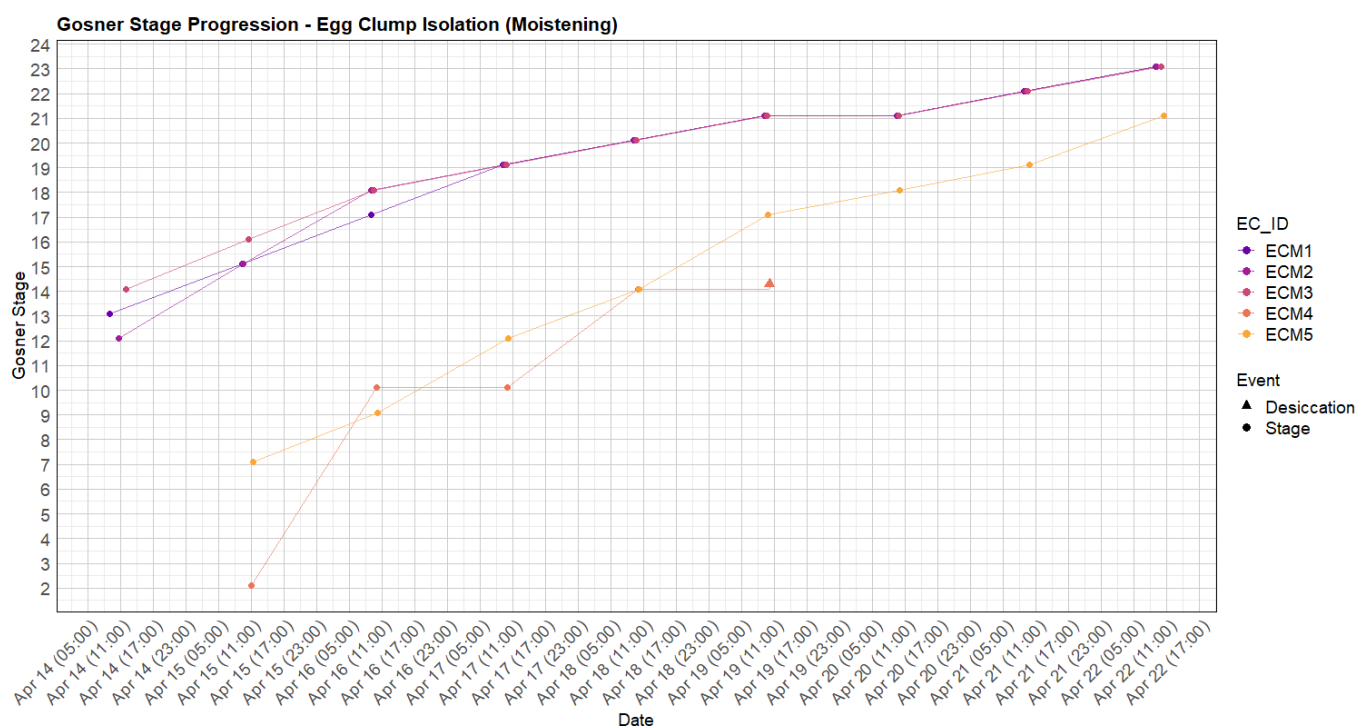


Figure 22. Time-series plot illustrating Gosner Stage progression over time for the April 2024 egg clump isolation experiment. The triangle symbol represents a desiccation event for the egg clump moistening treatment.

The time-series of the ECI treatment is illustrated in Figure 23. One isolated egg clump desiccated and one was predated upon by *Crematogaster* spp. ants, all others survived and continued with development at the experiment's conclusion.

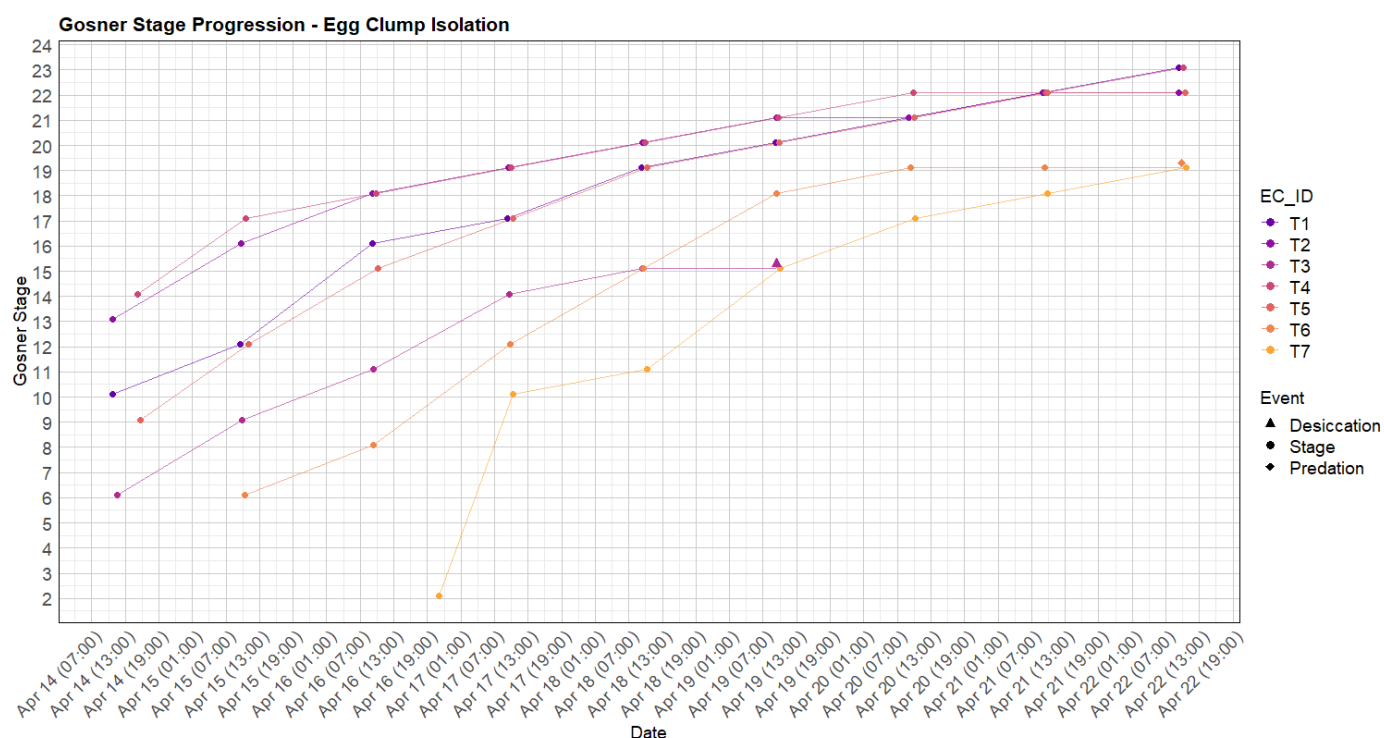


Figure 23. Time-series plot illustrating Gosner Stage progression over time for the April 2024 egg clump isolation experiment. The triangle symbol depicts a desiccation event and the square symbol represents a predation event for the egg clump isolation treatment.

The time-series of the control group for the April 2024 ECIE is shown in Figure 24. Only one egg clump was potentially preyed upon. Development for this egg clump underwent normal progression, until the egg clump completely vanished some time between 20 and 21 April. Mechanical damage to the attachment surface where the egg clump was positioned was evident, which leads to the assumption that the egg clump was predated upon by an unknown perpetrator.

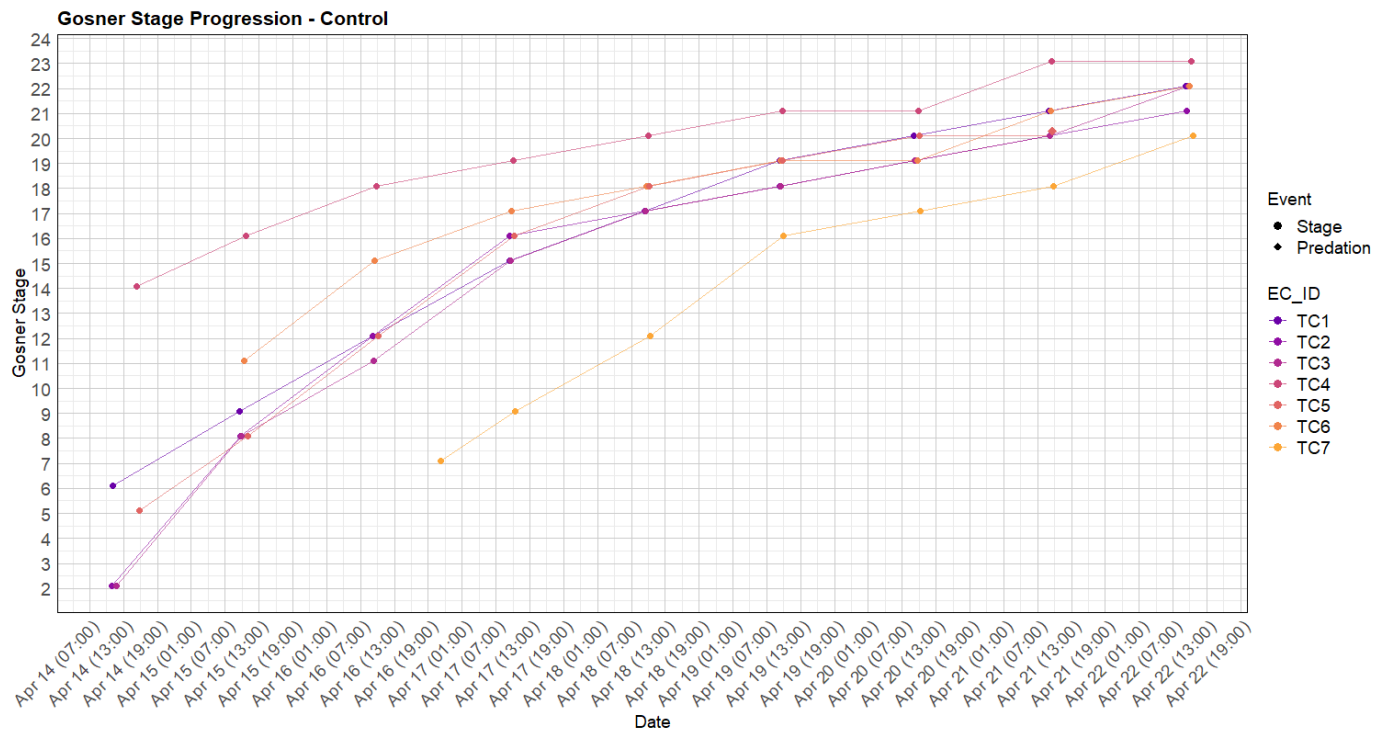


Figure 24. Time-series plot illustrating Gosner Stage progression over time for the April 2024 egg clump isolation experiment. A predation event is represented by a square for the control treatment.

Ordinal logistic regression models were fitted to the February 2024 dataset to examine the influence of temperature, relative humidity, height, attachment surface and level of shade on Gosner Stage in two experimental treatments: egg clump isolation (ECI) and Control. In the ECI treatment, none of the predictor variables significantly influenced Gosner Stage (all $p > 0.05$; Table 17). However, in the Control group, attachment surface type had a significant effect on Gosner Stage – egg clumps on leaves ($\beta = 2.74$, $p = 0.027$) and twigs ($\beta = 4.18$, $p = 0.011$) had strong positive associations with the progression of Gosner Stage over time than other attachment surfaces (Table 17). No significant effects were found for temperature, relative humidity, height or level of shade in either treatment in the February 2024 ECIE.

Table 17. Ordinal logistic regression model results for Gosner Stage (February 2024). Predictor variables for both egg clump isolation (ECI) and control groups are presented with estimates and p-values.

Predictor	Estimate (ECI)	p (ECI)	Estimate (Control)	p (Control)
Temperature	−0.023	0.946	0.200	0.672
Relative Humidity	0.327	0.391	0.281	0.557
Height	0.387	0.229	−0.458	0.383
Substrate (Leaf)	0.076	0.900	2.743	0.027
Substrate (Twig)	0.514	0.585	4.181	0.011
Substrate (Vine)	-	-	3.335	0.069
Shade (Light)	−0.189	0.745	−0.747	0.557
Shade (Partial)	−0.824	0.291	0.813	0.435

In the April 2024 ECIE, ordinal logistic regression was conducted to assess the influence of temperature, relative humidity, height, shade and attachment surface on Gosner Stage progression across three treatments: egg clump moistening (ECM), ECI and Control. The shade variable was not included in the ECM ordinal logistic regression model, as high multicollinearity with the attachment surface variable was detected through a preliminary model assessment which calculates generalised variance-inflation factors. The attachment surface variable was excluded from the ECI and Control ordinal logistic regression models, as there was only one level for this categorical variable (leaf) for both treatments. For these two variables that were not included in the logistic regression models, separate linear models were constructed to investigate their relationship with Gosner Stage, and both variables were found to have non-significant relationships with Gosner Stage across all treatments.

For the ECM treatment, temperature ($\beta = 1.428$, $p = 0.001$) and relative humidity ($\beta = 1.601$, $p < 0.001$) were shown to have positive and significant effects on Gosner Stage progression over time (Table 18). Height and attachment surface type had no significant effects on Gosner Stage progression.

Table 18. Ordinal logistic regression model results for Gosner Stage: Egg clump moistening treatment (April 2024). Predictor variables are presented with estimates, standard errors and p-values.

Predictor	Estimate	SE	p-value
Temperature	1.428	0.440	0.0012
Relative Humidity	1.601	0.457	0.0005
Height	0.437	1.500	0.7706

Attachment surface (Grass)	0.665	0.888	0.4536
Attachment surface (Leaf)	−0.055	3.198	0.9864

For the ECI treatment, temperature ($\beta = 0.646$, $p = 0.045$) and relative humidity ($\beta = 0.920$, $p = 0.005$) were also positive and significantly associated with Gosner Stage progression, whereas height and shade were not significant (Table 19). Gosner Stage progression is shown to be very intimately linked to relative humidity for the ECI treatment.

Table 19. Ordinal logistic regression model results for Gosner Stage: Egg clump isolation treatment (April 2024). Predictor variables are presented with estimates, standard errors and p-values.

Predictor	Estimate	se	p-value
Temperature	0.646	0.323	0.0454
Relative Humidity	0.920	0.331	0.0054
Height	−0.486	0.463	0.2941
Shade (Light)	0.434	1.332	0.7446
Shade (Partial)	1.022	0.767	0.1826

Similarly for the Control group, temperature ($\beta = 0.878$, $p = 0.003$) and relative humidity ($\beta = 0.922$, $p = 0.025$) remained positive and significant predictors of Gosner Stage progression over time, whereas the height and shade variables were not significant (Table 20). This group also showed that Gosner Stage progression was intimately linked with both temperature and relative humidity.

Table 20. Ordinal logistic regression model results for Gosner Stage: Control treatment (April 2024). Predictor variables are presented with estimates, standard errors and p-values.

Predictor	Estimate	SE	p-value
Temperature	0.878	0.299	0.0033
Relative Humidity	0.922	0.412	0.0254
Height	−0.814	0.709	0.2506

Shade (Light)	1.197	1.241	0.3349
Shade (Partial)	-0.260	0.619	0.675

To further investigate the relationship between recorded variables and their effect on Gosner Stage progression, power curves were constructed. These power curves gave indications of the rate of development (Gosner Stage) of eggs as time progressed. The power curve constructed for the February 2024 ECIE is shown in Figure 25, indicating developmental rate for the ECI ($R^2 = 0.347$) and Control ($R^2 = 0.316$) treatments. These curves illustrate that clear differences in developmental rates were evident between the two groups. The ECI group rose sharply in developmental rate at the start of the experiment, but decreased in developmental rate as time progressed. The Control group also rose sharply in developmental rate at the start of the experiment and kept increasing as time progressed.

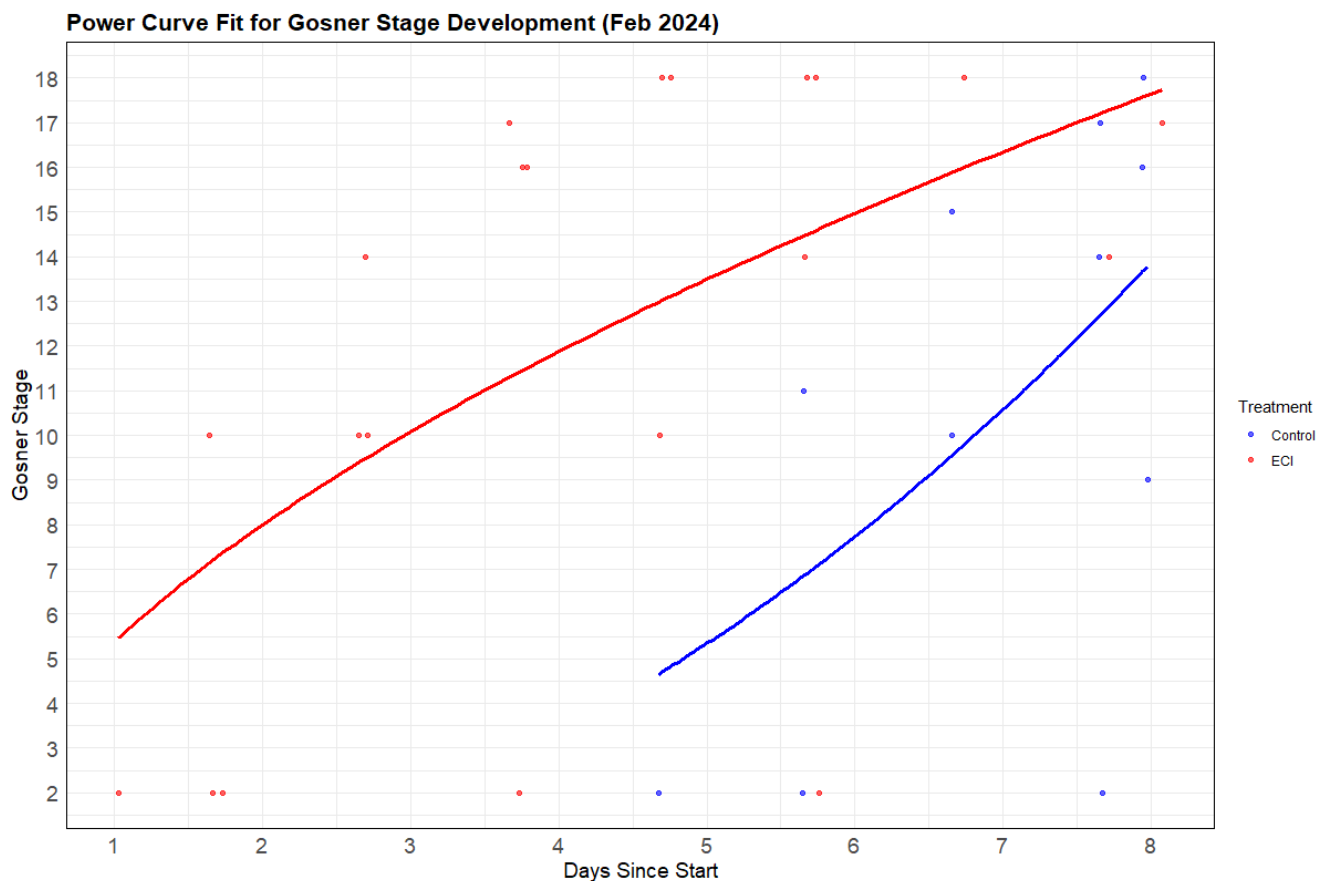


Figure 25. Power curve of egg clump isolation (ECI) and control treatments of the February 2024 egg clump isolation experiment, illustrating developmental rate (Gosner Stage) over time (Days Since Monitoring Started).

The developmental rate for the ECM, ECI and Control treatments of the April 2024 ECIE is illustrated in Figure 26. These curves indicate subtle differences in the developmental rate between the three groups. The ECM group showed a rapid increase in developmental rate over time ($R^2 = 0.822$). The ECI group showed a slower initial increase in developmental rate when compared with the ECM and Control groups ($R^2 = 0.829$). The Control group showed a rapid initial increase in developmental rate and slowed as time progressed ($R^2 = 0.928$).

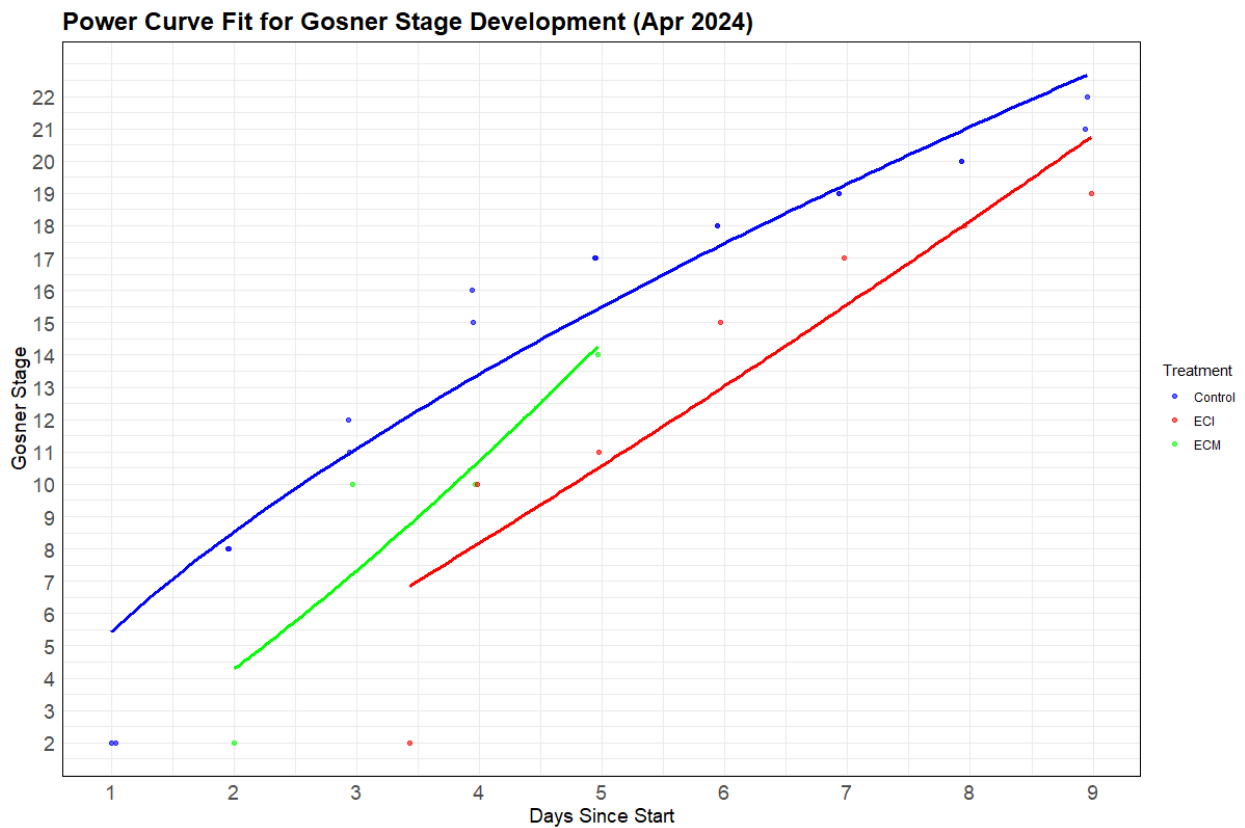


Figure 26. Power curve of egg clump moistening (ECM) treatment, egg clump isolation (ECI) treatment and control treatment from April 2024 egg clump isolation experiment illustrating developmental rate (Gosner Stage) over time (Days Since Start).

3.2 Acoustic monitoring

3.2.1 Call profiles

Call profiles were identified from Zoom F1 directional recordings using Raven Pro acoustic analysis software, where multiple individual calls were isolated and spectral measurements were taken. Seven distinctive call types were identified throughout the study period (Table 21).

Table 21. Identified call types and number of calls for each call type used for spectral measurements and analyses.

Call type	Number analysed
Click	30
Croak	21
Distress call	2
Squeak	22
Squeak-trill	2
Trill	30
Two-click	30

The “click” call type (Figure 27 and Figure 28) is the advertisement call, employed by male *N. bonebergi*. This is the most common call type heard amongst reproductively active males.

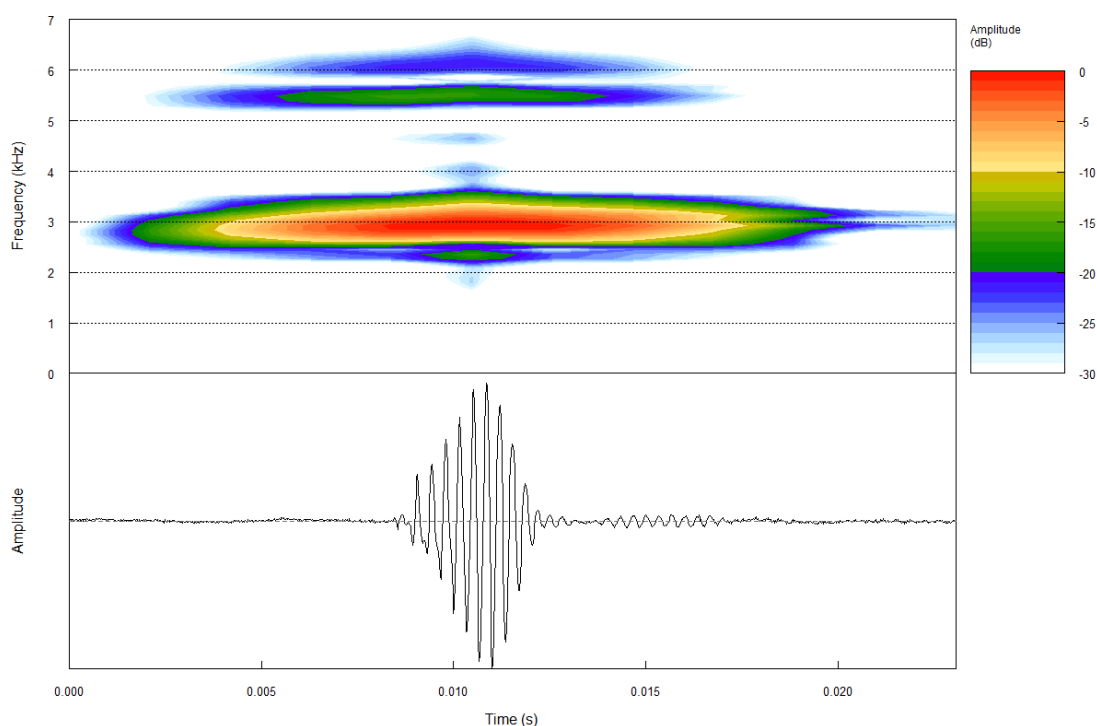


Figure 27. Call profile of the "Click" call type, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram.



Figure 28. QR code linking to audio of the "Click" call type.

The “croak” call type is used infrequently and is very soft (Figure 29 and Figure 30). This call type was mostly observed to be utilised in agonistic contexts in between other call types.

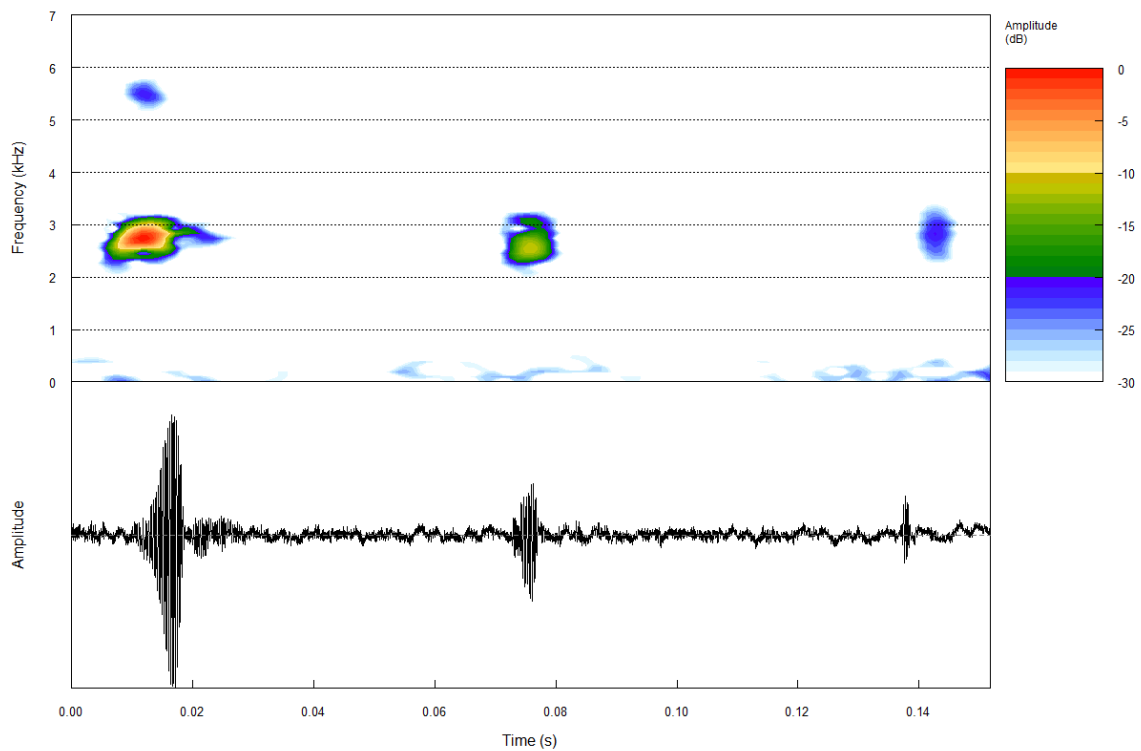


Figure 29. Call profile of the "Croak" call type, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram.



Figure 30. QR code linking to audio of the "Croak" call type.

The “distress call” call type (Figures 31–33) was also rarely recorded, except during terminal agonistic interactions between males (brawling). This call is usually emitted loudly in a defensive context when a male is being assailed by another.

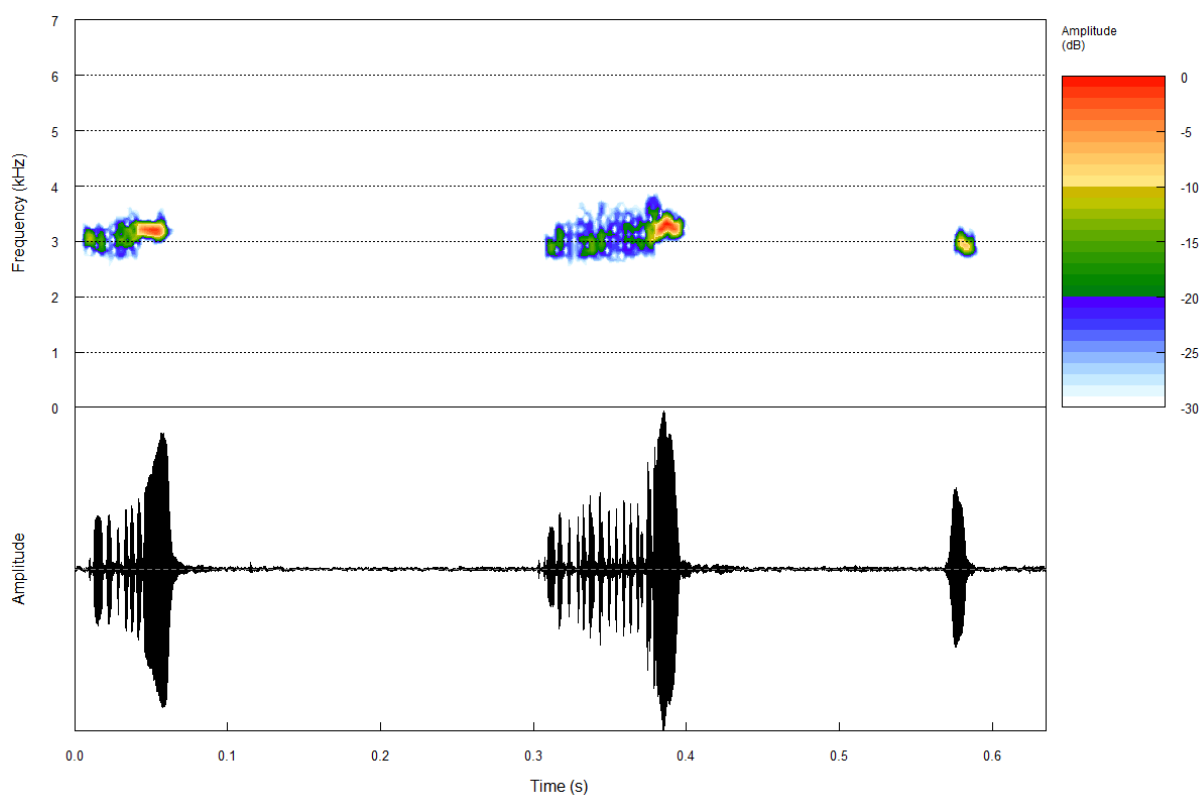


Figure 31. Call profile of the "Distress call" call type, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram.

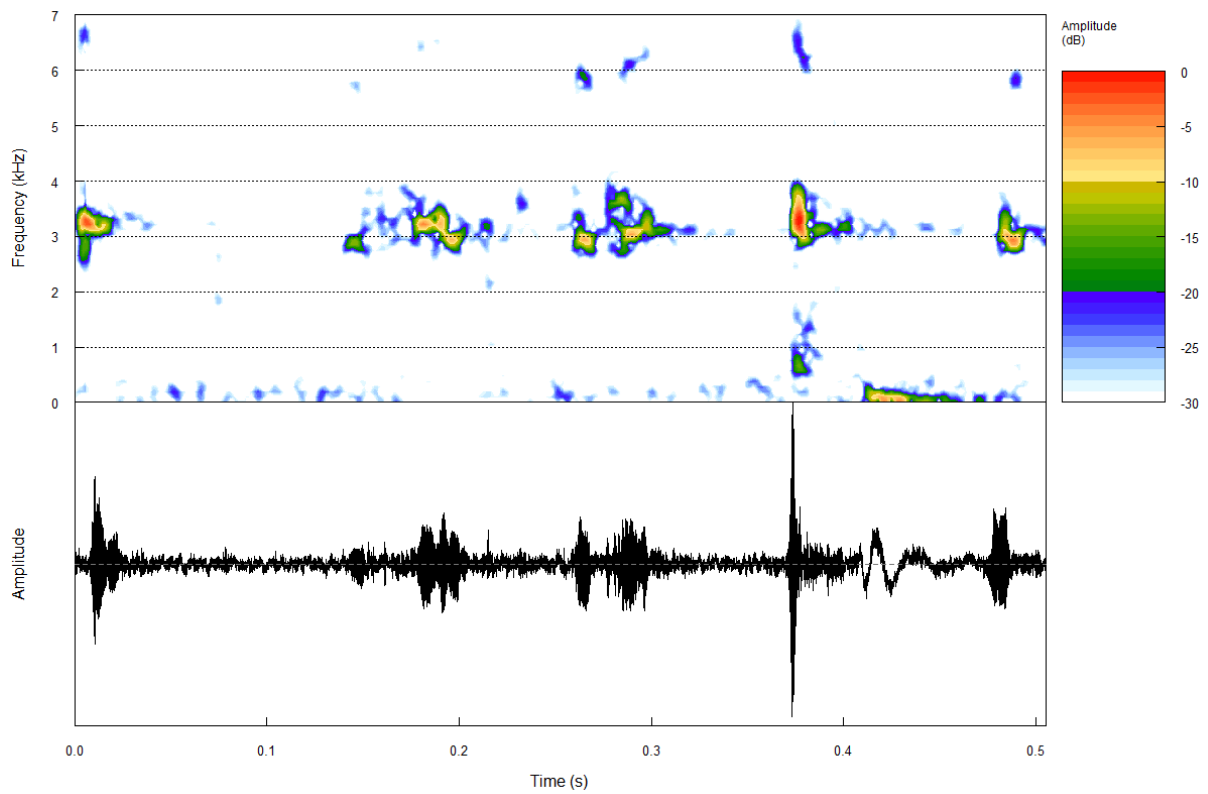


Figure 32. Call profile of the "Distress call" call type, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram.



Figure 33. QR code linking to audio of the "Distress call" call type.

The “squeak” call type (Figures 34–37) is a distinctive call typically employed by males in agonistic interactions with conspecifics. This is the loudest of all recorded call types, and often varies in length. This call type often sounds like a bottle of wine being uncorked.

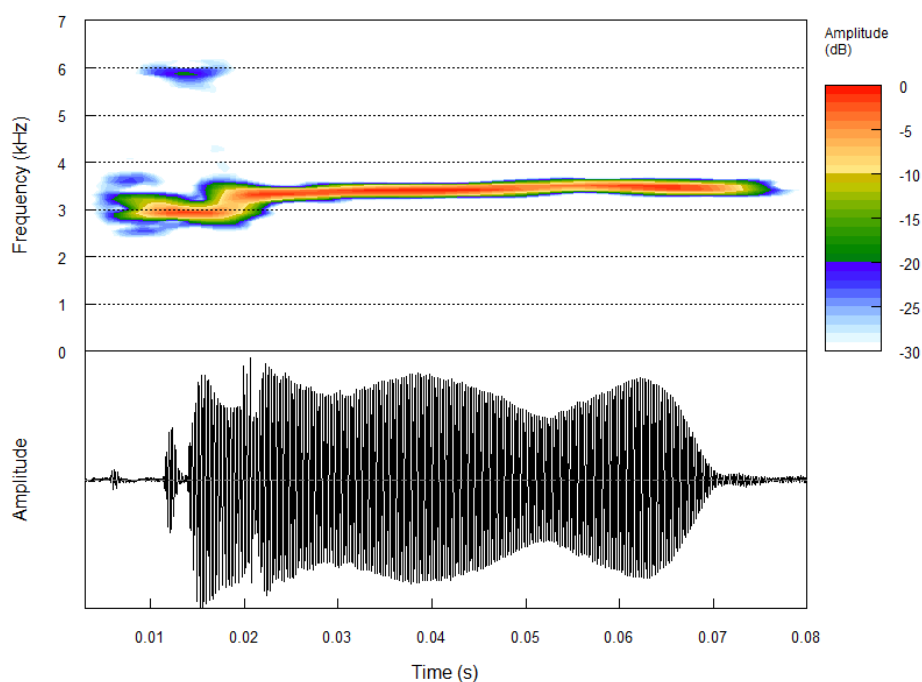


Figure 34. Call profile of the "Squeak" call type, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram.

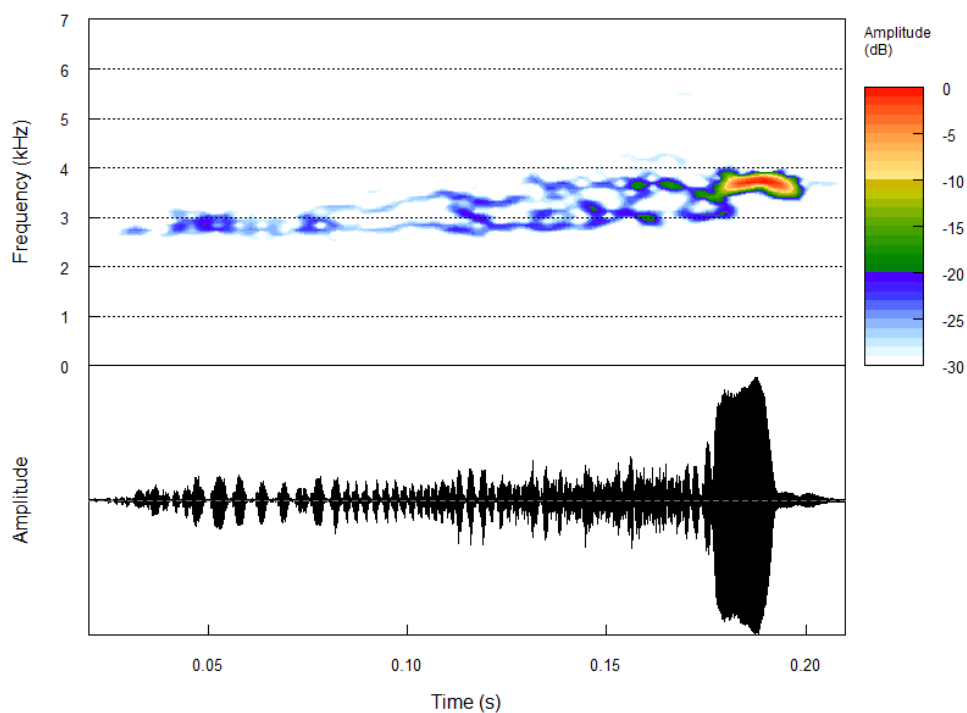


Figure 35. Call profile of the "Squeak" call type, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram.

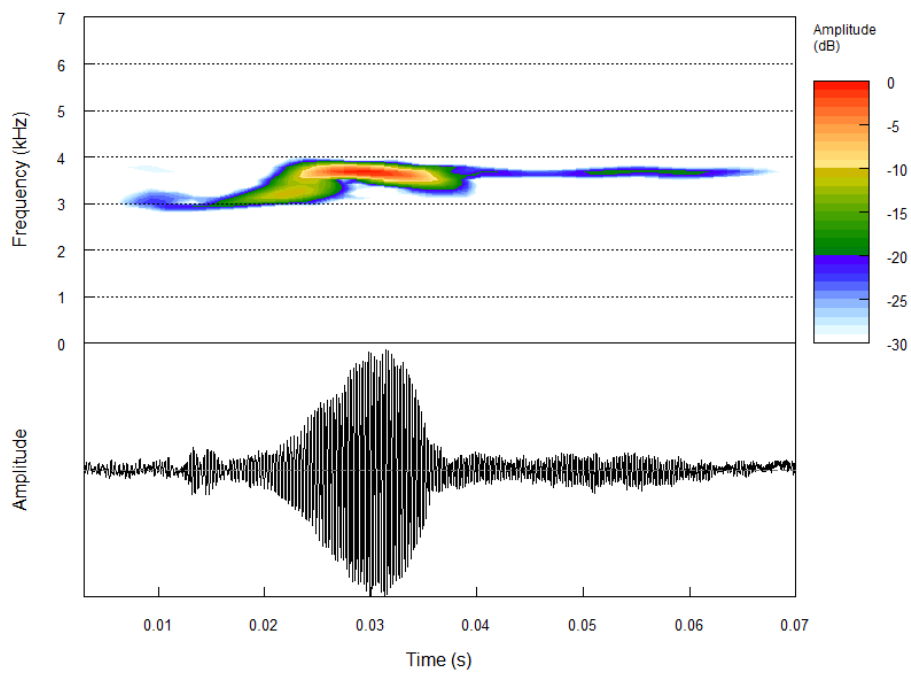


Figure 36. Call profile of the "Squeak" call type, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram.



Figure 37. QR code linking to audio of the "Squeak" call type.

The “squeak-trill” call type (Figures 38–40) is seldom heard but is a “trill” call type directly followed by the “squeak” call type. This call type is also usually employed in agonistic interactions.

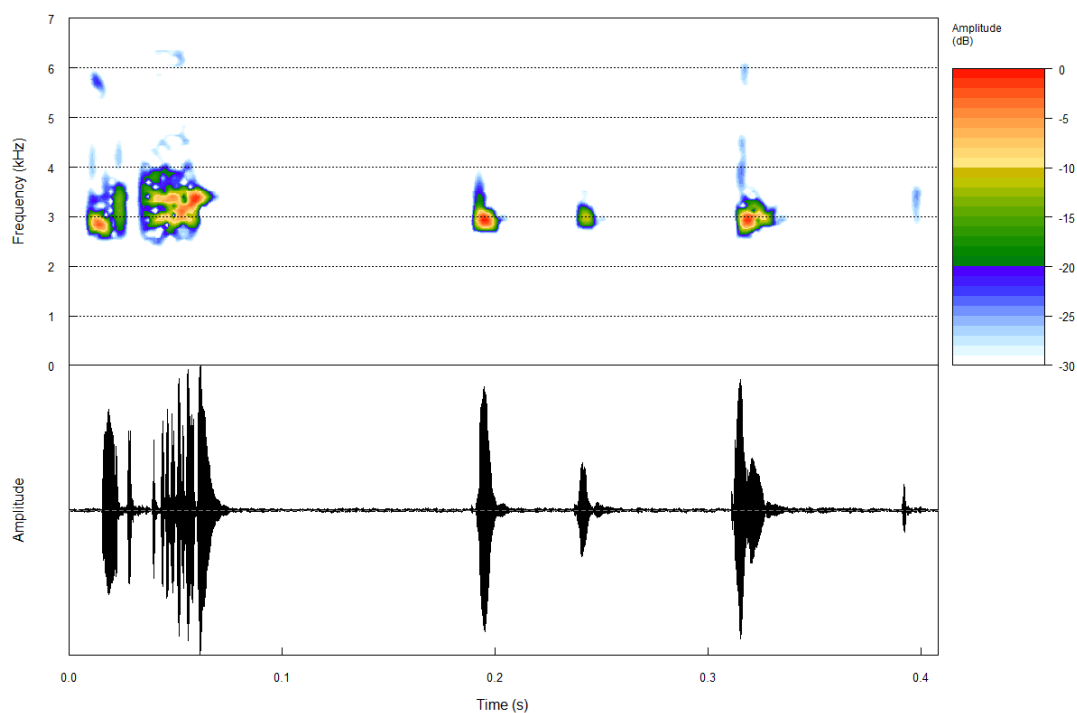


Figure 38. Call profile of the "Squeak-trill" call type, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram.

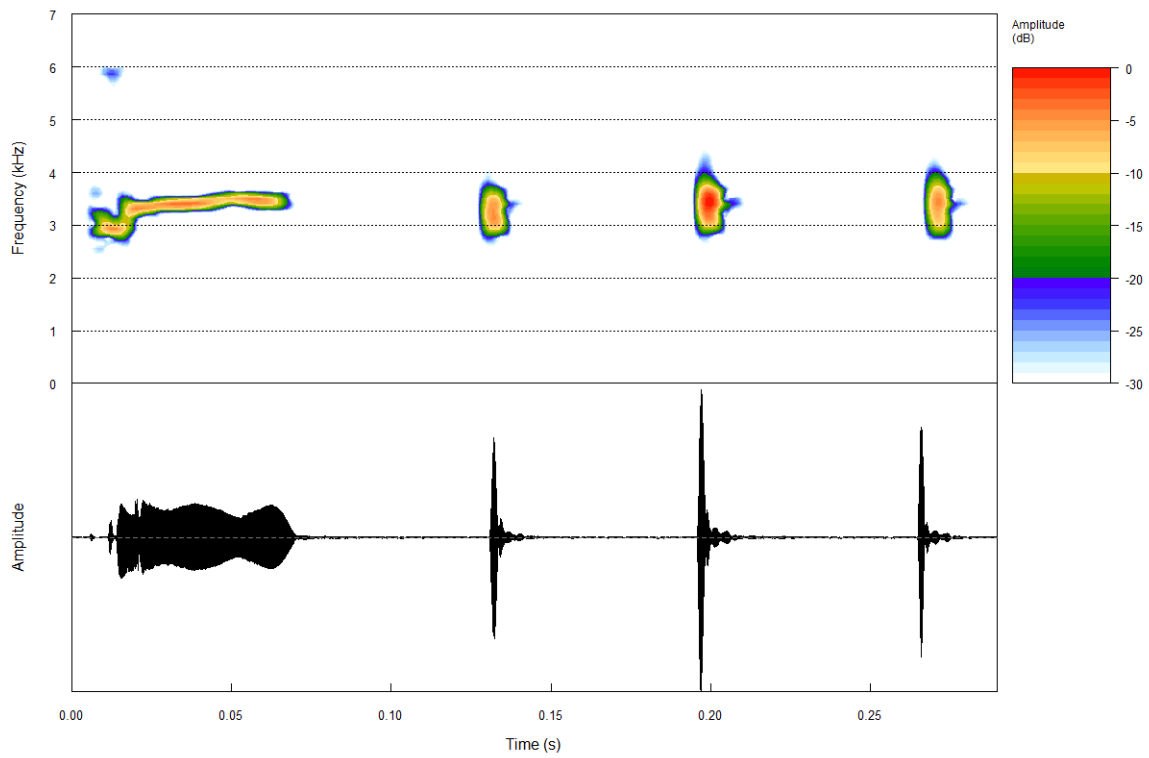


Figure 39. Call profile of the "Squeak-trill" call type, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram.



Figure 40. QR code linking to audio of the "Squeak-trill" call type.

The “trill” call type is also employed in agonistic interactions with conspecifics (Figures 41–44). This call type varies drastically in length and usually causes conspicuous rapid bulging of male vocal sacs.

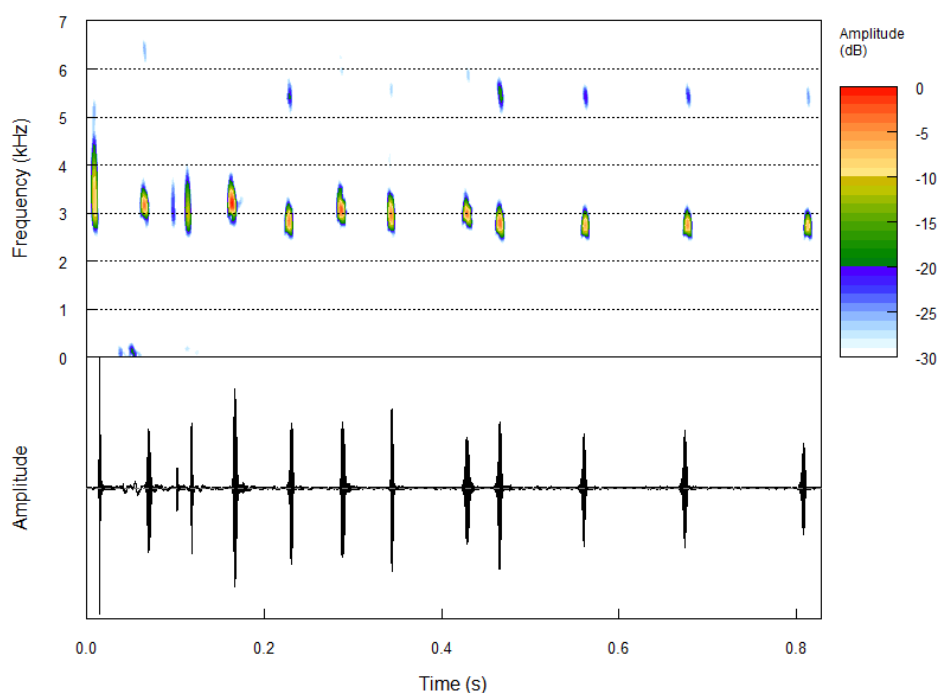


Figure 41. Call profile of the "Trill" call type with 13 individual notes, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram.

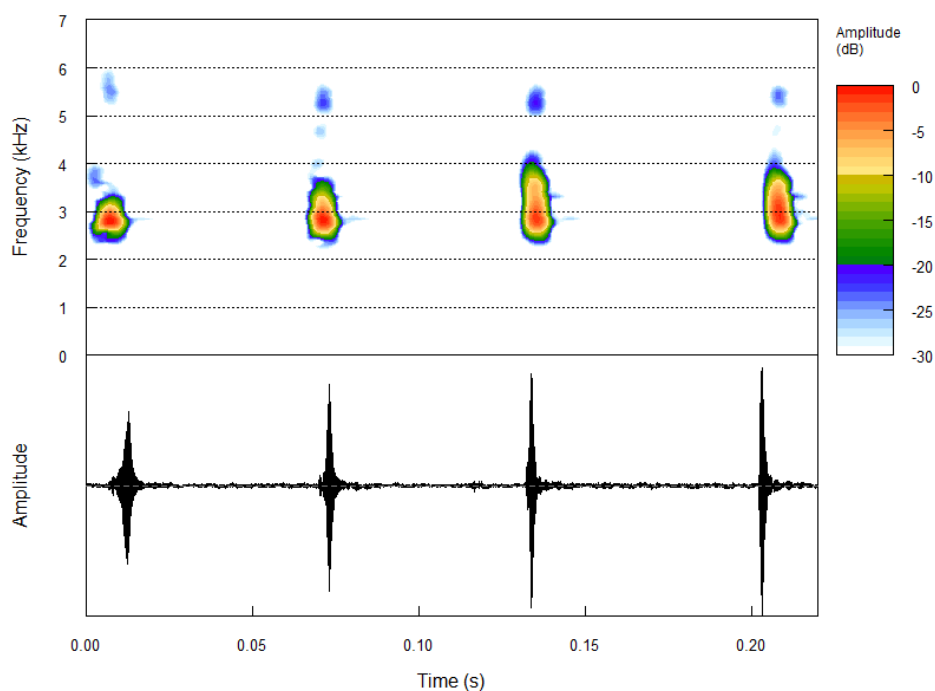


Figure 42. Call profile of the "Trill" call type with three individual notes, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram.

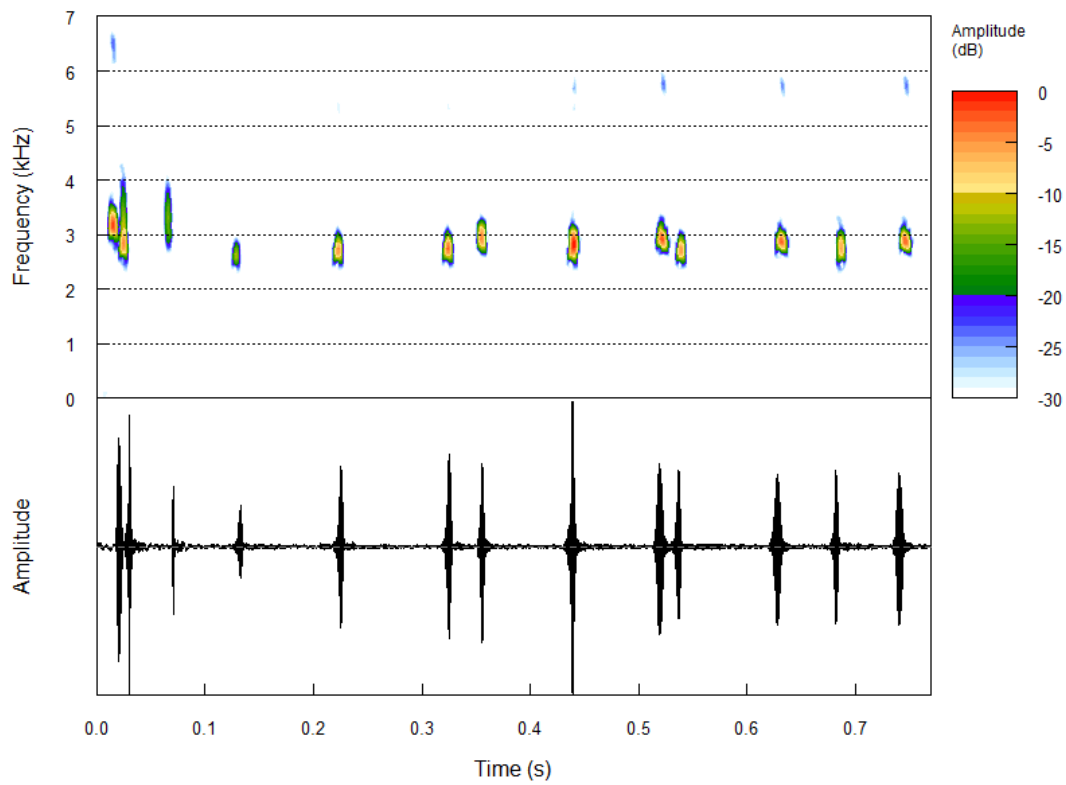


Figure 43. Call profile of the "Trill" call type with 13 individual notes, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram.



Figure 44. QR code linking to audio of the "Trill" call type.

The “two-click” call type (Figure 45 and Figure 46) is akin to the advertisement “click”. This call type is emitted in quick succession relative to the advertisement call.

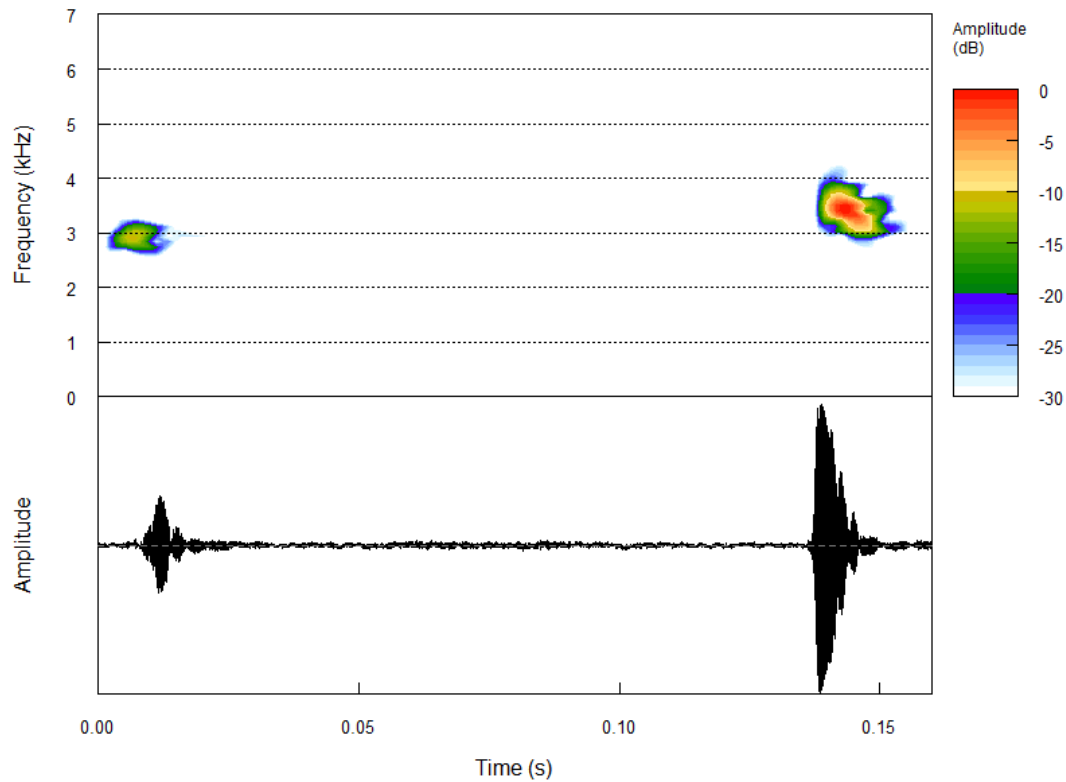


Figure 45. Call profile of the "Two-click" call type, illustrating the frequency spectrum (spectrogram) and corresponding oscillogram.



Figure 46. QR code linking to audio of the "Two-click" call type.

These call types were annotated and measured in Raven Pro, subsequently generating call parameters in selection tables from which summary statistics for dominant frequency (kHz), dominant frequency range (kHz), call duration (s), note duration (s) and note quantity were obtained in R and RStudio (Table 22). Note quantity for certain call types (squeak, squeak-trill, croak and distress calls) could not be accurately estimated, as these call types are pulsatile – consisting of fast amplitude modulation without intermittent silence, showing no clearly countable peaks.

Table 22. Call parameters (means and standard deviations) of different call types.

Call parameters	Click (mean ± SD)	Two-click (mean ± SD)	Trill (mean ± SD)	Squeak (mean ± SD)	Squeak-trill (mean ± SD)	Croak (mean ± SD)	Distress (mean ± SD)
Dominant frequency (kHz)	2.996 ± 0.208	2.945 ± 0.199	3.082 ± 0.818	3.148 ± 0.273	3.163 ± 0.026	2.772 ± 0.132	2.931 ± 0.110
Minimum dominant frequency (kHz)	2.775 ± 0.435	2.3 ± 0.337	2.203 ± 0.746	2.02 ± 0.710	2.016 ± 0.796	1.909 ± 0.269	1.734 ± 0.663
Maximum dominant frequency (kHz)	3.063 ± 0.244	3.331 ± 0.823	4.588 ± 1.825	4.982 ± 2.439	5.953 ± 3.580	3.904 ± 1.082	6.891 ± 1.326
Dominant frequency range (kHz)	0.288 ± 0.487	1.031 ± 0.960	2.384 ± 1.645	2.962 ± 2.573	3.938 ± 0.333	1.996 ± 1.117	5.156 ± 1.989
Call duration (s)	0.028 ± 0.022	0.125 ± 0.056	0.421 ± 0.203	0.268 ± 0.214	0.454 ± 0.097	0.265 ± 0.069	0.877 ± 0.493
Note quantity (n)	1	2	3–13	NA	NA	NA	NA
Note duration (s)	0.028 ± 0.022	0.125 ± 0.056	0.421 ± 0.203	0.268 ± 0.214	0.454 ± 0.097	0.265 ± 0.069	0.877 ± 0.493

3.2.2 Passive acoustic monitoring

The call parameters obtained from statistical analysis of the call types were used in Raven Pro's Band-Limited Energy Detector (BLED) Batch Detection tool to identify the various *N. bonebergi* calls across all SM4 recordings from 14 April 2023 to 21 April 2024. These results were compiled with temperature data collected by the SM4 and the obtained monthly rainfall data in Table 23.

Table 23. Band-Limited Energy Detector output from the Raven Pro Batch Detection tool. Outputs were organised in monthly categories with mean temperature (°C), total call counts per month (n) and monthly rainfall (mm).

Year	Month	Mean Temperature (°C)	Call Count (n)	Rainfall (mm)
2023	Apr	19.7	332574	42
2023	May	17.0	495041	225
2023	Jun	15.2	382538	116
2023	Jul	14.6	409109	16
2023	Aug	16.1	533396	22
2023	Sept	17.4	583172	66
2023	Oct	17.4	414931	235
2023	Nov	19.9	917759	88
2023	Dec	20.0	944295	199
2024	Jan	21.2	518498	103
2024	Feb	21.9	746395	25
2024	Mar	21.8	841338	61
2024	Apr	19.4	527750	189

The total number of calls detected each month is illustrated in Figure 47, however, both April 2023 and April 2024 may have an underestimated number of calls, as all days in both months were not fully recorded. Peaks in calling activity were clearly evident in November, December, February and March. From previous data, we may assume that April of both years would also have shown peaks in calling activity.

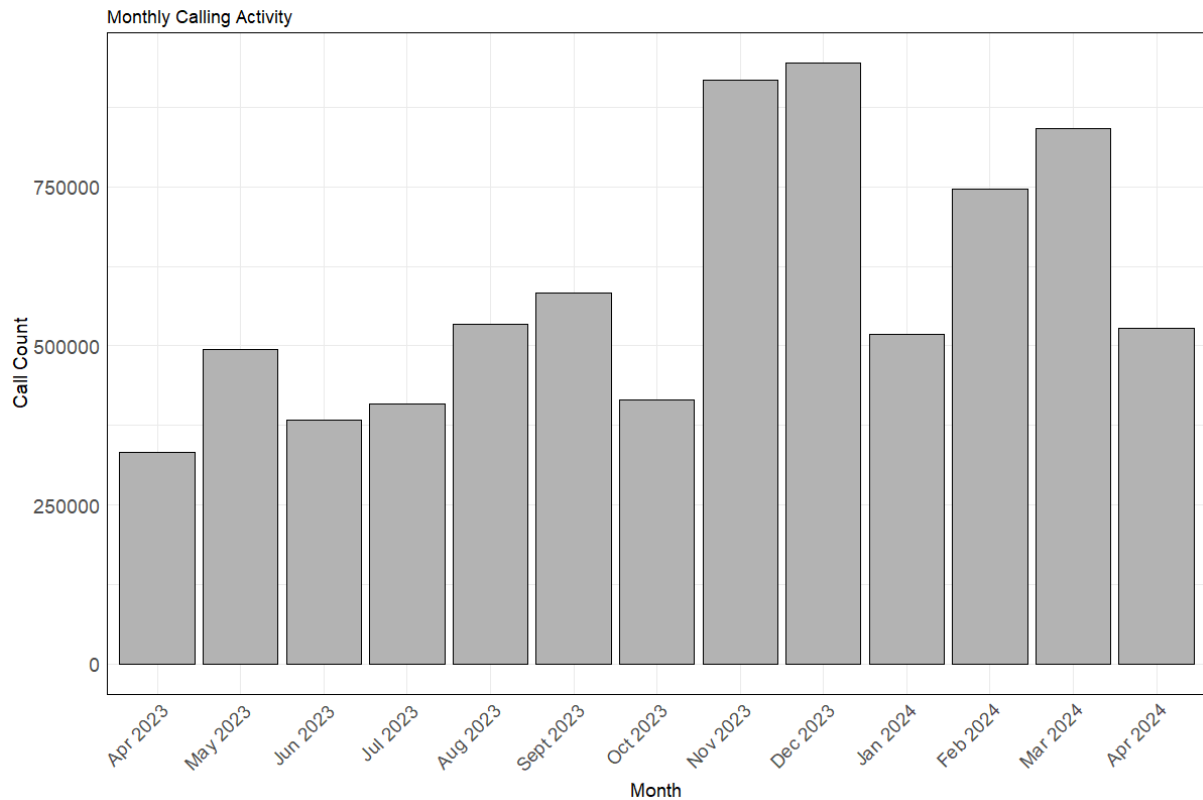


Figure 47. Bar graph of total number of calls detected throughout each month from April 2023 to April 2024.

To gain insight into the calling activity of *N. bonebergi* over the 24-h period, the mean number of calls (with standard deviations) are summarised in Table 24 with mean hourly temperature (°C). Calling activity increased from 19:00 and peaked around midnight. Calling activity decreased slightly in the early morning hours and reached another peak around dawn (05:00–07:00).

Table 24. The number of calls detected over 24 hours (mean and standard deviation) with mean temperature (°C).

Hour	Call count (mean)	Call count (SD)	Temperature (°C) (mean)
00:00	1075	1031	17.7
01:00	1045	990	17.5
02:00	1019	920	17.3
03:00	965	847	17.2
04:00	998	857	17.0
05:00	998	847	16.9
06:00	1112	789	16.8
07:00	1024	583	16.8

08:00	827	549	17.1
09:00	732	542	17.7
10:00	716	534	18.5
11:00	696	522	19.4
12:00	646	517	20.1
13:00	604	485	20.6
14:00	596	507	20.9
15:00	571	488	20.9
16:00	596	537	20.7
17:00	915	653	20.1
18:00	841	773	19.5
19:00	866	825	19.0
20:00	903	896	18.6
21:00	933	841	18.3
22:00	963	880	18.1
23:00	1028	937	17.9

Hourly calling activity for the entire dataset is illustrated in Figure 48. Clear increases in calling activity could be observed around dusk and dawn.

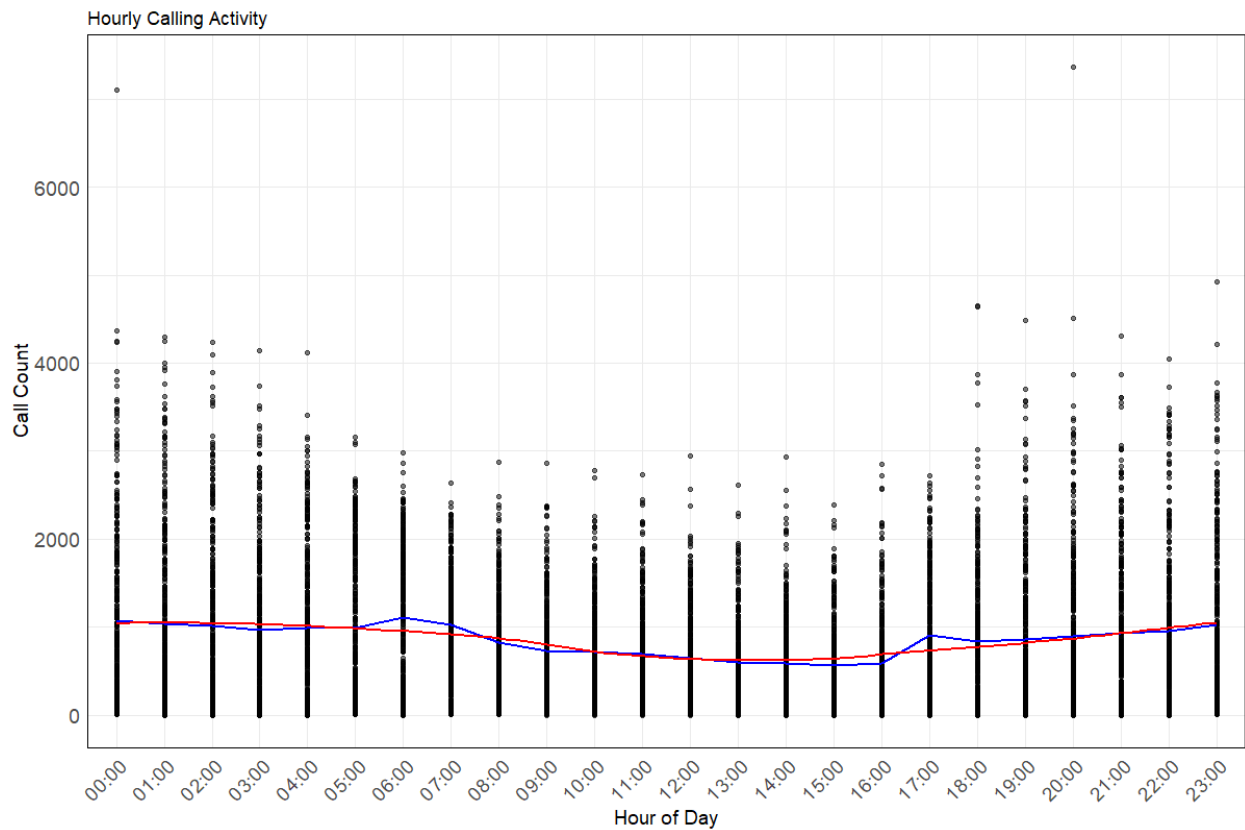


Figure 48. Dot plot of call counts per hour with a blue line indicating the summary of calls for each hour, and a red line as a smoothed approximation of the blue summary line.

Monthly rainfall was plotted against the total number of calls per month and fitted with a regression line to show the correlation between these variables in Figure 49. The slope of the regression line indicates that no correlation existed between rainfall and calling activity on the monthly scale.

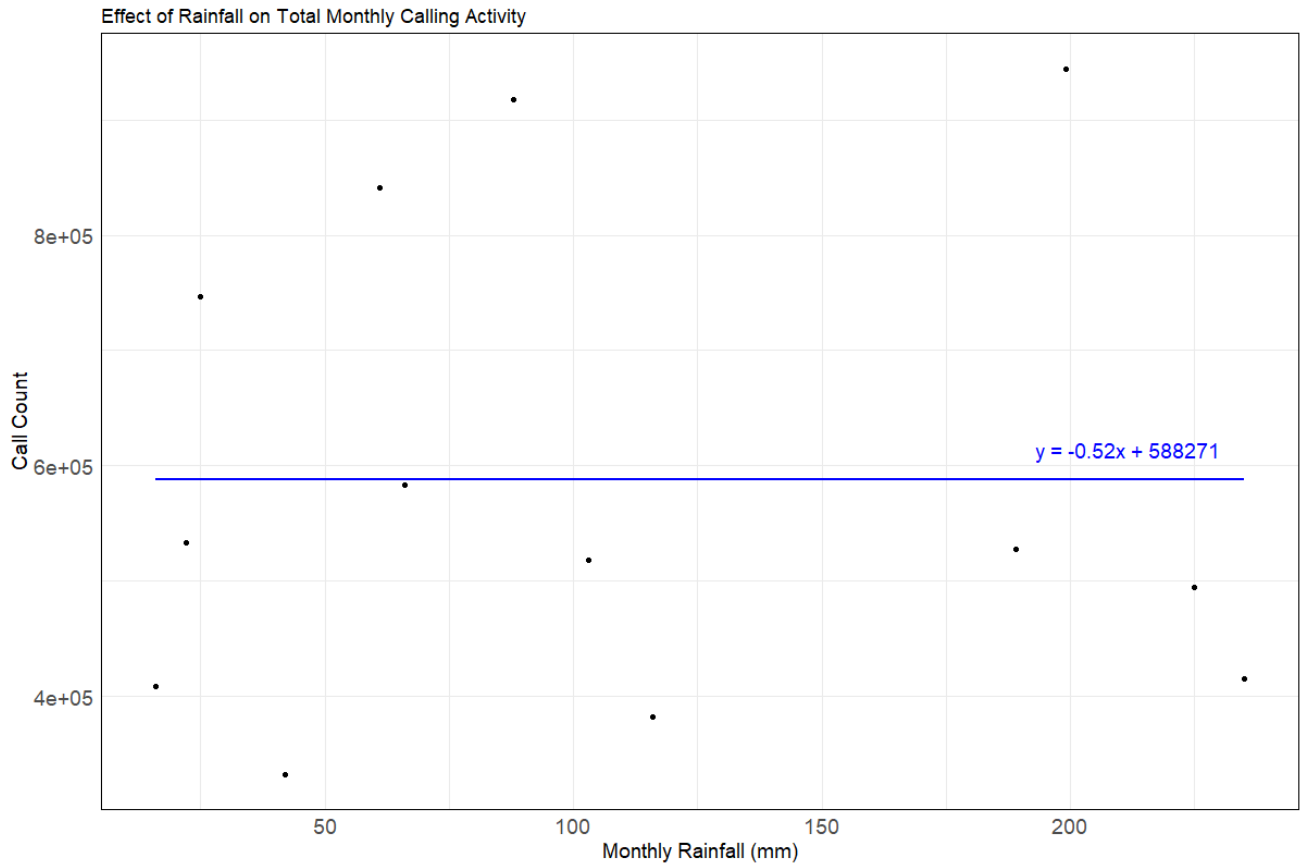


Figure 49. Scatterplot of total calls detected per month vs monthly rainfall (mm) fitted with a regression line.

A similar approach was used when plotting mean daily temperature (°C) against the total number of calls per day (Figure 50). The slope of the fitted regression line shows that a strong positive correlation existed between mean daily temperature and daily calling activity, indicating that calling activity increased with ambient temperature at breeding sites ($r^2 = 0.188$).

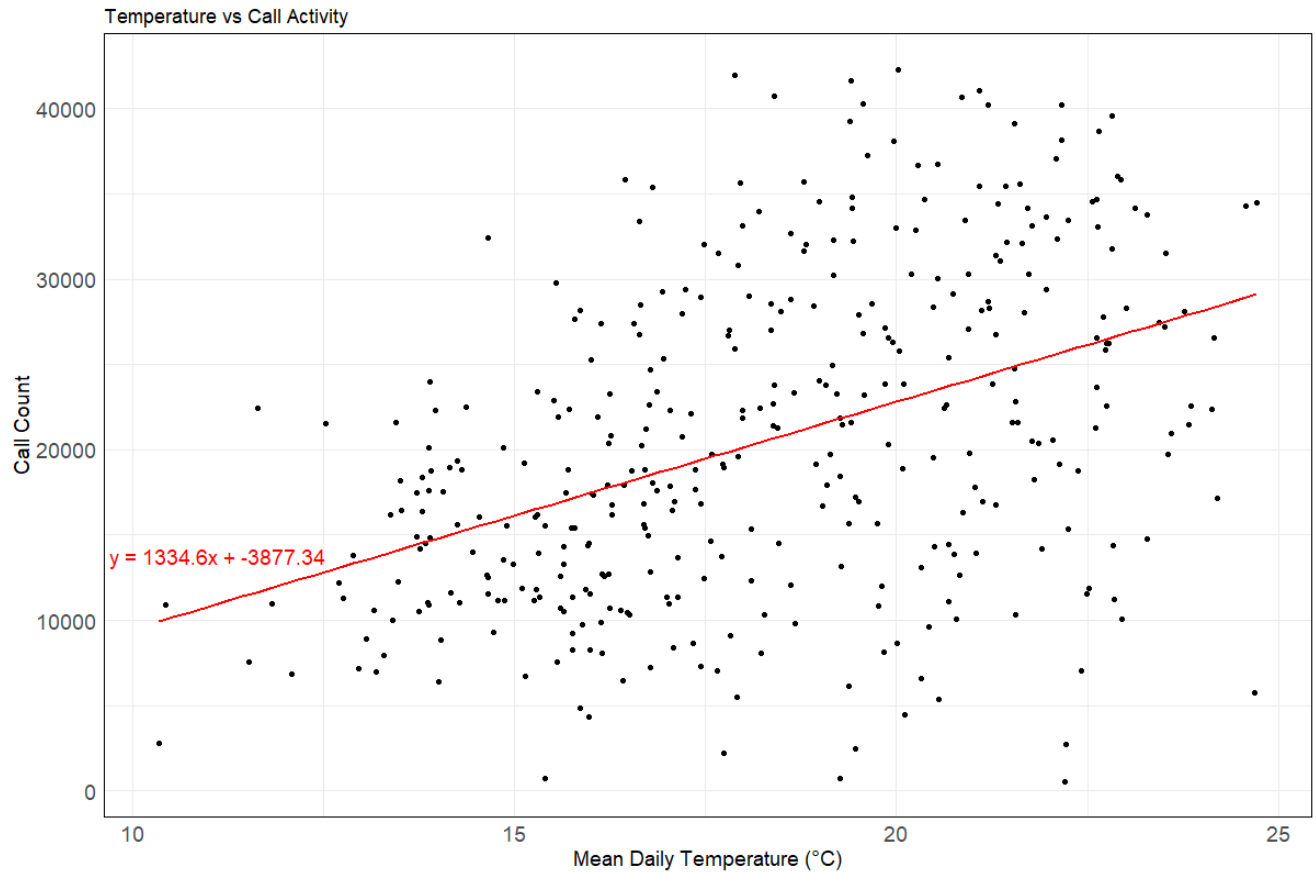


Figure 50. Scatterplot of the total number of calls detected per day vs mean daily temperature (°C) fitted with a regression line ($y = 1334.6x + -3877.34$).

Centre frequency (Hz) of each detected call is illustrated in Figure 51, where the means of these centre frequencies were plotted per month. Changes in centre frequencies over time may indicate changes in the soundscape throughout the year at this breeding site and may also be related to calling activity within and outside of the *N. bonebergi* breeding season.

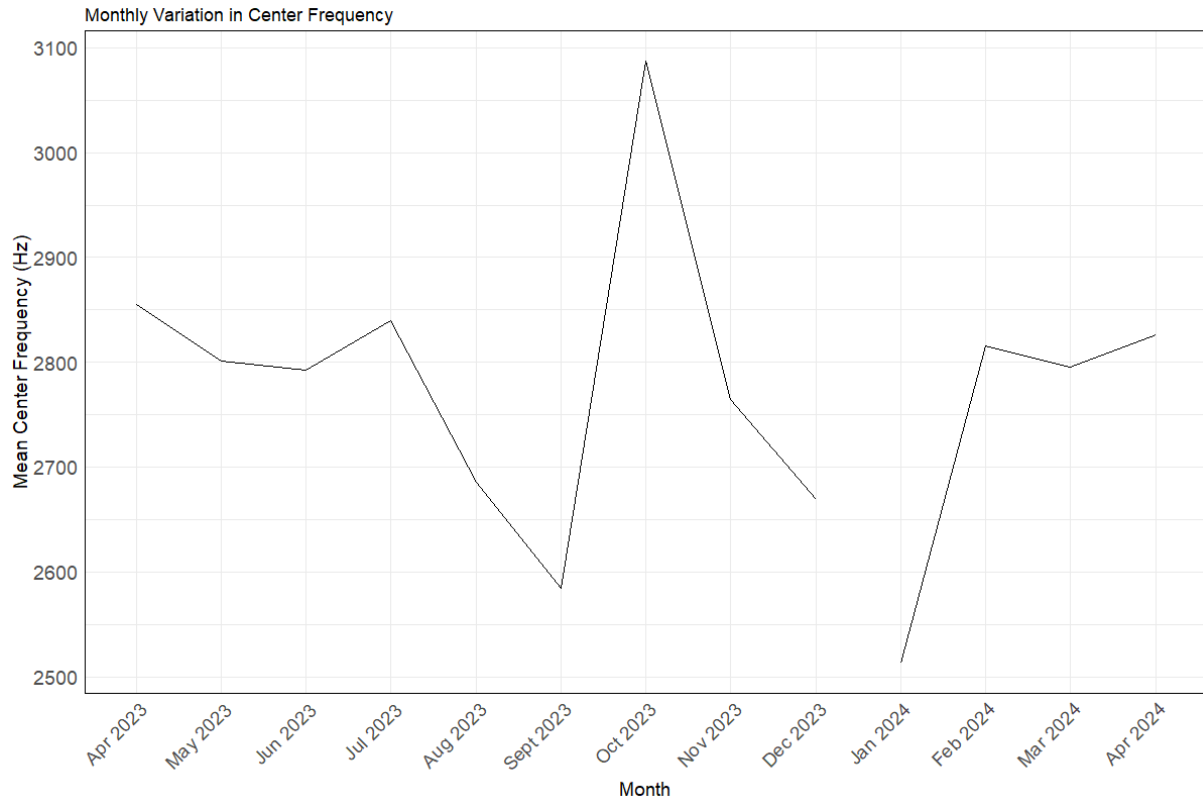


Figure 51. Line graph of mean centre frequency (Hz) detected for calls per month from April 2023 to April 2024.

3.3 Observations of behaviour

Observed behaviours were recorded and classified into four categories: visual behaviours, dual visual behaviours, calling behaviours and multimodal behaviours. Frequency tables for all recorded categorical variables were constructed in R and RStudio for the visual behaviours category, which includes behavioural category (Table 25) and visual behaviours recorded for each sex (Table 26) as well as the substrates on which visual behaviours were recorded (Table 27).

Table 25. Behavioural category and number of times each category was recorded for each sex.

Type	n	n = female	n = male
Agonistic	193	11	182
Defensive	29	7	22
Parental care	24	24	0
Reproductive	161	101	60
Undetermined	30	28	2
Total	437	171	266

Table 26. Visual behaviours recorded for each sex, describing the context in which these behaviours were recorded for each sex.

Behaviour	n	n = female	n = male	Context
Abdomen contraction	54	31	23	Parental care (F) Reproduction (F, M)
Accidental amplexus	2	0	2	Reproductive (M)
Amplexus attempt	15	0	15	Reproductive (M)
Body contortion	6	2	4	Defensive (F) Reproductive (M)
Body lowering	4	0	4	Low-intensity territorial defensive (M)
Body orientation	10	0	10	Low-intensity territorial defensive (M)
Both legs kicking	17	1	16	Terminal defensive (F) Terminal territorial agonistic (M)
Brawling	1	0	1	High-intensity territorial agonistic (M)
Bumping	2	0	2	High-intensity territorial agonistic (M)
Chasing	6	0	6	High-intensity territorial agonistic (M)
Cloaca movement	3	3	0	Reproductive (F)

Defensive urination	4	2	2	High-intensity defensive (F, M)
Egg clump rubbing	11	11	0	Parental care (F)
Eye poking	1	1	0	High-intensity defensive (F)
Face wiping	2	2	0	Reproductive (F)
Head tilt	16	16	0	Low-intensity defensive (F)
Leg kicking	41	5	36	Terminal defensive (F) Terminal territorial agonistic (M)
Leg rubbing	25	25	0	Reproductive (F)
Limb lifting	8	8	0	Undetermined (F)
Mouth gaping	3	3	0	Undetermined (F)
Physical contact	18	2	16	Low-intensity defensive (F) Low-intensity territorial agonistic (M)
Pushing	15	1	14	Low-intensity territorial agonistic (M)
Spread limbs	24	0	24	Low-intensity territorial agonistic/ low-intensity defensive (M)
Toe trembling	111	57	54	Alerting signal/ ritualised behaviour (F, M)
Topping	30	2	28	Low-intensity defensive (F) Low-intensity territorial agonistic (M)
Upright posture	2	0	2	Low-intensity territorial agonistic/ low-intensity defensive (M)
Urination	2	0	2	Undetermined/ reproductive (M)
Vocal sac bulging	5	0	5	Low-intensity territorial agonistic (M)
Total	437	171	266	

Table 27. Substrates on which visual behaviours were recorded.

Substrate	n
Fern	83
Leaf	14
Palm	80
Riverbed	4
<i>Scadoxus</i>	140
Vine	116
Total	437

Recorded visual behaviours were divided into behavioural categories in Figure 52. The same recorded visual behaviours were divided by sex in Figure 53.

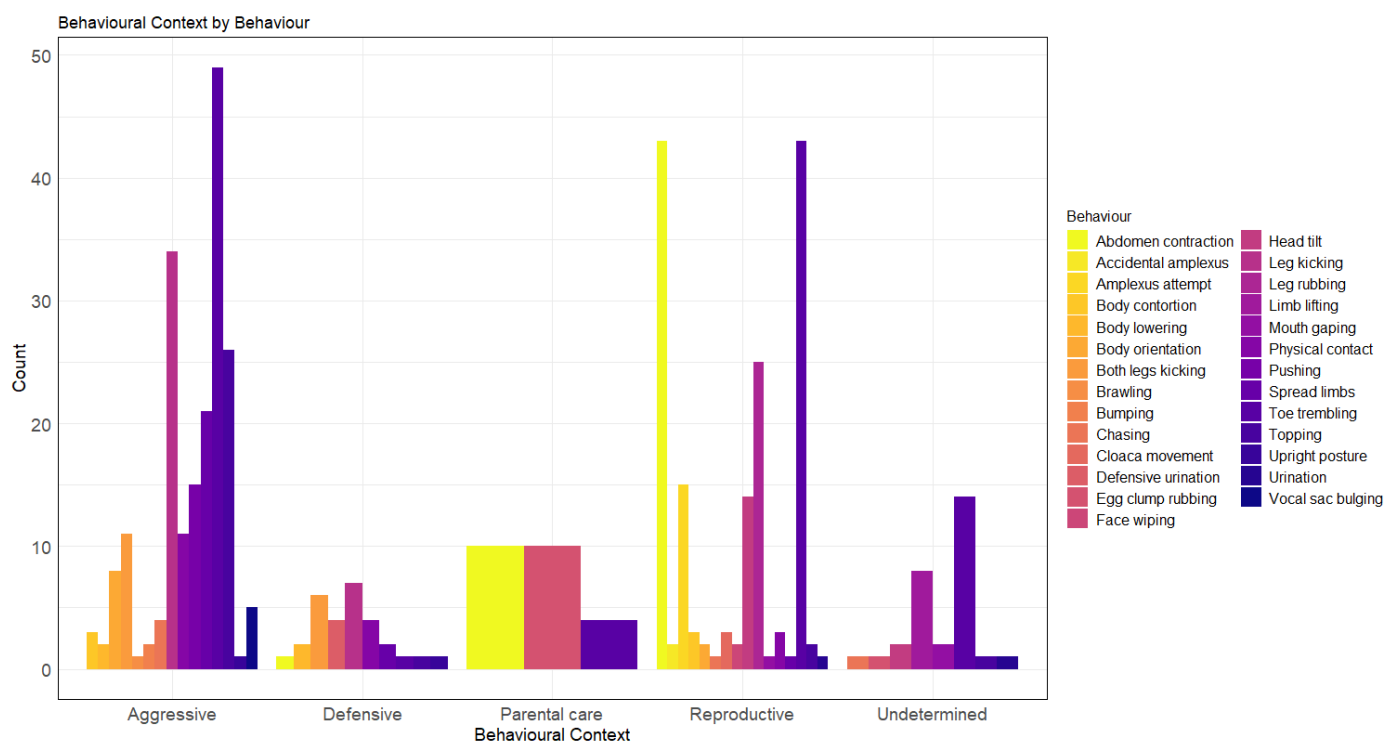


Figure 52. Bar graphs dividing the number of recorded visual behaviours into behavioural categories.

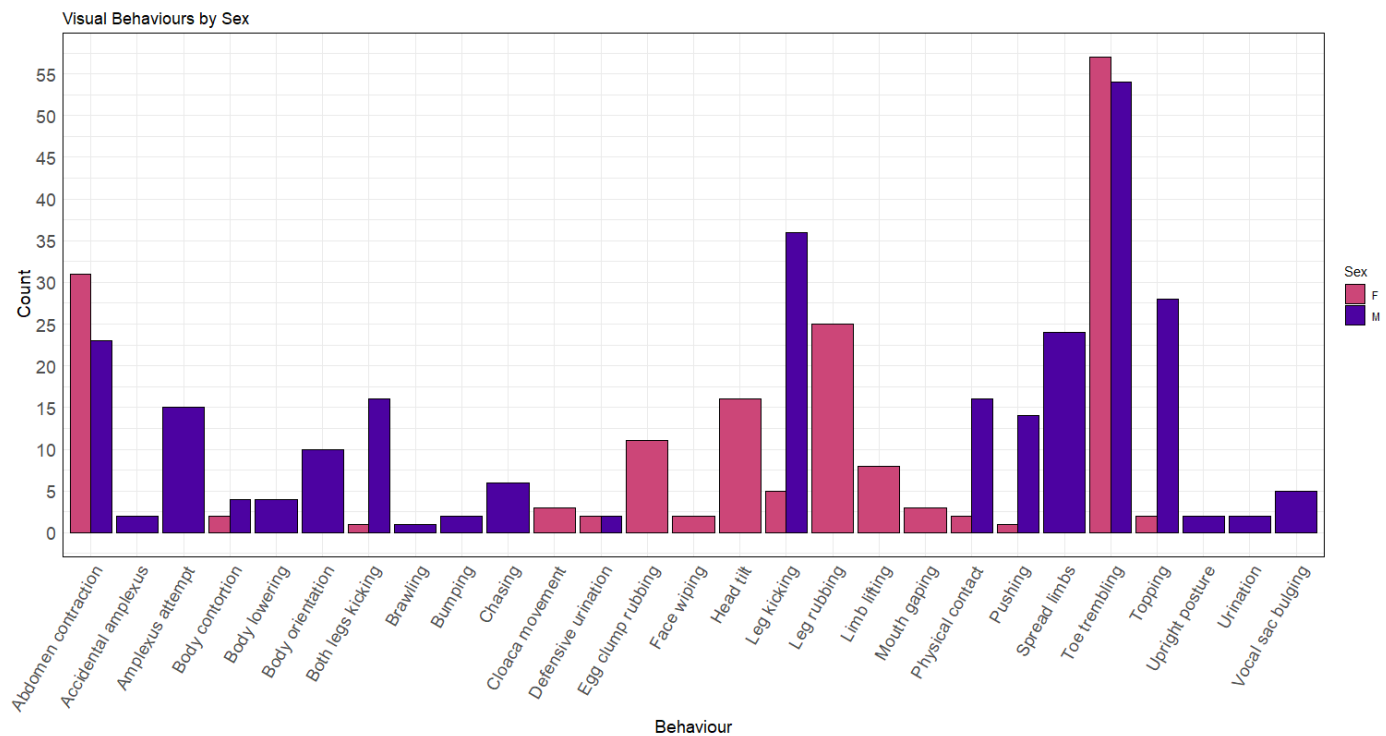


Figure 53. Bar graph of all recorded visual behaviours employed by sexes.

Visual behaviour categories are illustrated by sex using a mosaic plot (Figure 54). The proportions of each behavioural category corresponding to either male (M) or female (F) indicate the relative number of observations made in each of these behavioural categories. Males predominantly exhibited aggressive behaviours and no behaviours related to parental care. Defensive behaviours were mostly recorded from males attempting to defend themselves from competing males. Female defensive behaviours were mostly recorded when amplexus were attempted on unreceptive females. Females exhibited more reproductive behaviours, and these behaviours were mostly recorded while females were in amplexus or laying eggs. Numerous additional behaviours observed in females were not attributable to any specific behavioural category, such as limb lifting, toe trembling and mouth gaping, as these behaviours were recorded in the absence of other individuals in their immediate vicinity.

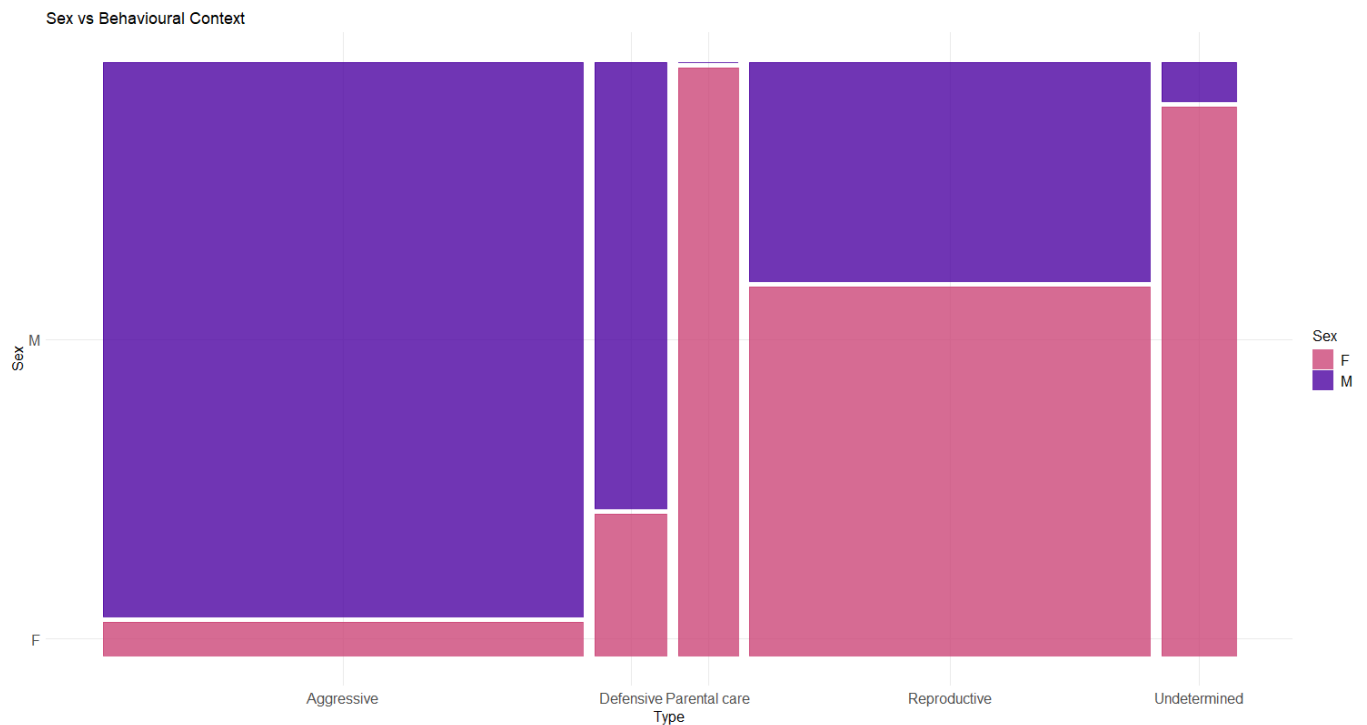


Figure 54. Mosaic plot of visual behaviour categories divided by sex.

Dual visual behaviours recorded during field observations were also recorded in one frequency table, showing which visual behaviour was observed to be used simultaneously with another visual behaviour (Table 28). Toe trembling was the most common observed visual behaviour used in conjunction with other visual behaviours.

Table 28. Visual behaviours observed to be used conjunctively, the sex which employed the behaviours, social contexts in which behaviours occurred and the behavioural categories. The social context category shows which sex facilitated the dual behaviour on which other sex or individual/s.

Visual behaviour	Conjunctive behaviour	n	Sex	Social context	Behavioural category
Toe trembling	Body contortion	2	M F	M-M F-M-M	Reproductive Defensive
Toe trembling	Body lowering	1	M	M-M	Aggressive
Toe trembling	Body orientation	1	M	M-M	Aggressive
Toe trembling	Head tilt	3	F	F-M F	Defensive Undetermined
Toe trembling	Limb lifting	2	F	F	Undetermined
Toe trembling	Physical contact	1	M	M-M	Aggressive

Toe trembling	Spread limbs	6	M	M-M	Aggressive
Toe trembling	Topping	4	M	M-M	Aggressive
Leg kicking		2	M	M-M	Aggressive
Spread limbs		3	M	M-M	Aggressive
Physical contact	Upright posture	1	M	M-M	Aggressive

From all 136 minutes of video recordings, 311 calls were identified (Table 29) in respect to their behavioural categories (Table 30, Figure 55). Most calls were used in agonistic aggressive or defensive categories. The “click” call type was the most used call in reproductive contexts. “Distress” calls were predominantly used in defensive contexts, while the “squeak-trill” call type was used exclusively in aggressive contexts.

Table 29. Call types identified during field observations.

Call type	n	Proportion per time (%)
Click	61	19.6
Croak	66	21.2
Distress	21	6.8
Squeak	71	22.8
Squeak-trill	5	1.6
Trill	50	16.1
Two-click	37	11.9
Total	311	100

Table 30. Behavioural category of call types observed in the field.

Call type behavioural category	n
Aggressive	232
Defensive	45
Reproductive	34
Total	311

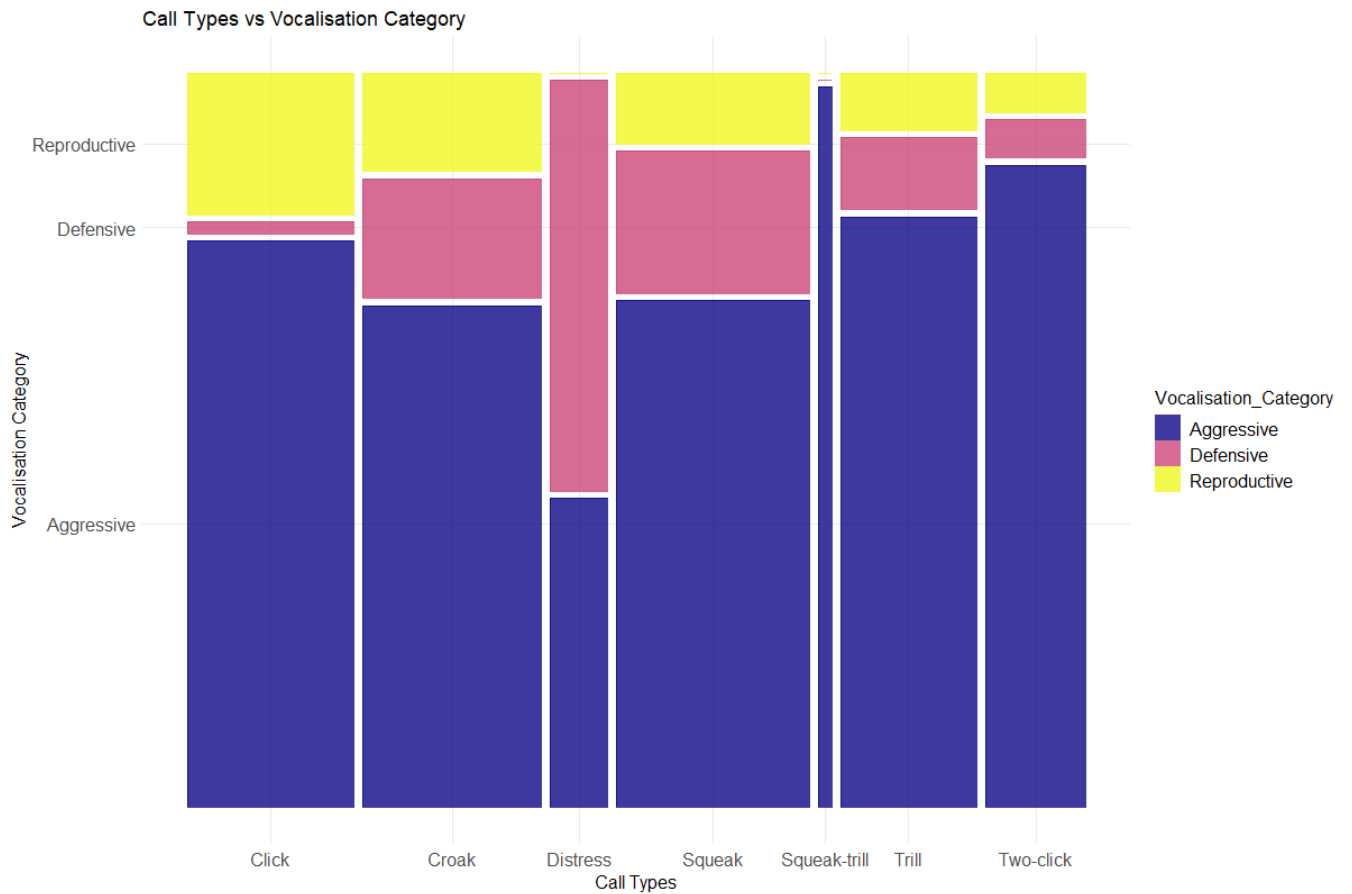


Figure 55. Mosaic plot of call types divided into vocalisation category.

The substrates from which call types were identified in video recordings are summarised in Table 31. These substrates from which call types were identified are illustrated in Figure 56.

Table 31. Substrates on which calling occurred at the study site, identified from video recordings.

Substrate	n
Fern	75
Palm	88
<i>Scadoxus</i>	80
Vine	68
Total	311

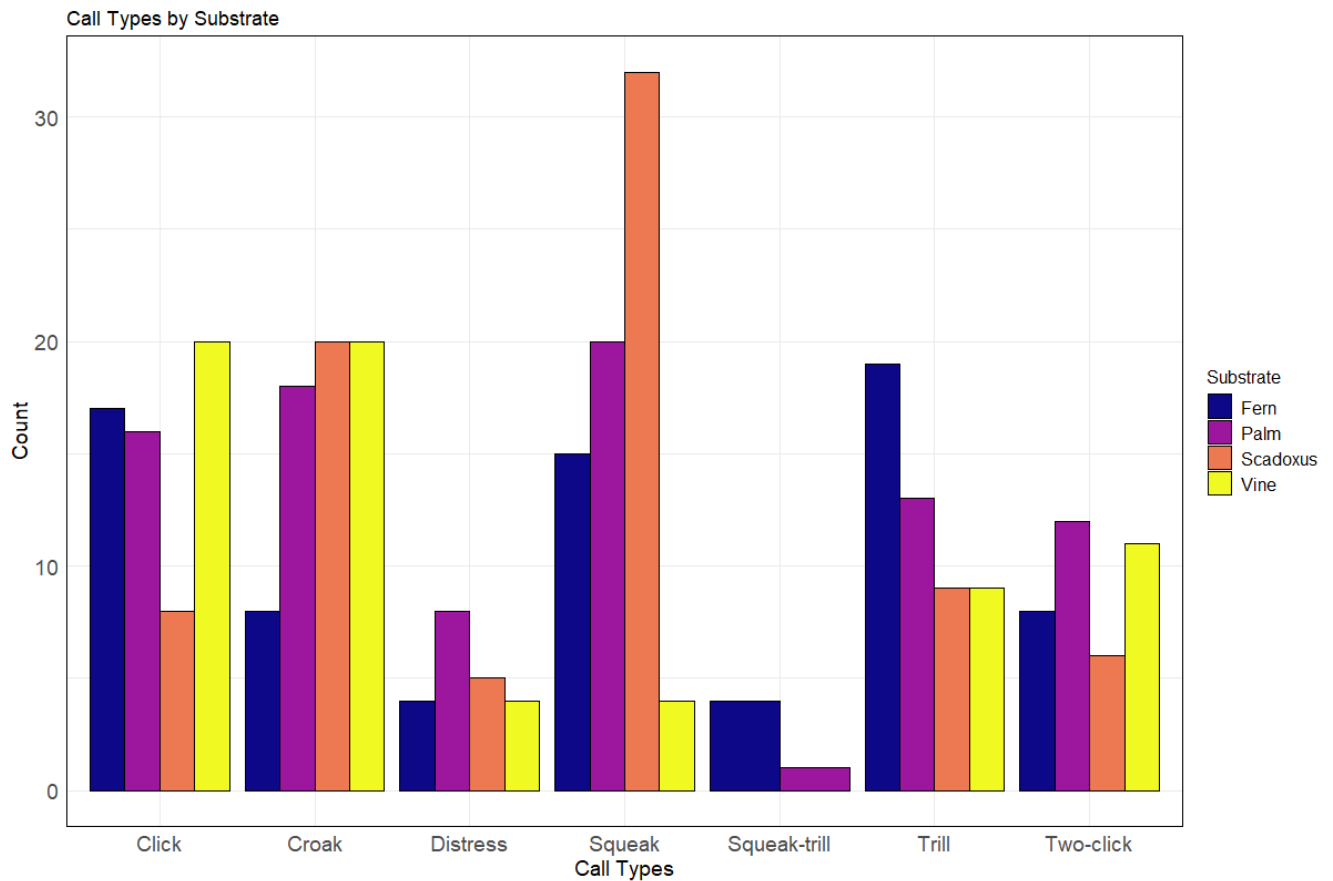


Figure 56. Bar graph of call types as recorded on different substrates.

A total of 86 instances of multimodal behaviours were identified from field observations (Table 32, Figure 57), and only one of these instances were of an unreceptive female on her way to moisten an egg clump. This was also the only recorded instance of a vocalisation emitted by a female, as a click was utilised while the female was trembling her toes in a defensive context in response to a male that attempted amplexus. Only seven of these multimodal behaviours were in a reproductive behavioural context, and these include: Click-toe trembling, employed by a male while not surrounded by any individuals; Croak-toe trembling, employed by the same male in the same context; Squeak-physical contact, employed by a male that approached and touched a female attending to her egg clump (the male emitted three squeaks while making physical contact); Squeak-toe trembling, employed by a male on the edge a palm leaf while not near any other individual; Click/Croak/Trill-body contortion, employed by a male that positioned himself on top of a female, but not yet in amplexus, while seemingly tugging at the female beneath him. All other multimodal behaviours were employed in aggressive contexts.

Table 32. Multimodal behaviours (call type emitted with visual behaviours) recorded through field observations. 'n' is the total number of times a call type was recorded with the corresponding visual behaviours, and 'n2' is the number of times a specific visual behaviour is recorded with the corresponding call type.

Call type	n	Visual behaviour	n2
Click	20	Toe trembling	8
		Topping	3
		Physical contact	3
		Defensive urination	1
		Leg kicking	1
		Chasing	1
		Spread limbs	1
		Body contortion	1
		Body lowering	1
Croak	19	Toe trembling	7
		Spread limbs	5
		Leg kicking	2
		Pushing	2
		Body contortion	1
		Topping	1
		Vocal sac bulging	1
Distress	6	Both legs kicking	1
		Brawling	1
		Leg kicking	1
		Spread limbs	1
		Topping	1
		Vocal sac bulging	1
Squeak	22	Topping	5
		Body orientation	4
		Toe trembling	4
		Physical contact	3
		Both legs kicking	2
		Brawling	1
		Body orientation	1
		Pushing	1
		Chasing	1
Squeak-trill	2	Spread limbs	1

		Pushing	1
Trill	14	Toe trembling	5
		Topping	2
		Spread limbs	2
		Body contortion	1
		Body orientation	1
		Physical contact	1
		Pushing	1
		Vocal sac bulging	1
Two-click	3	Pushing	1
		Spread limbs	1
		Toe trembling	1
Total	86		

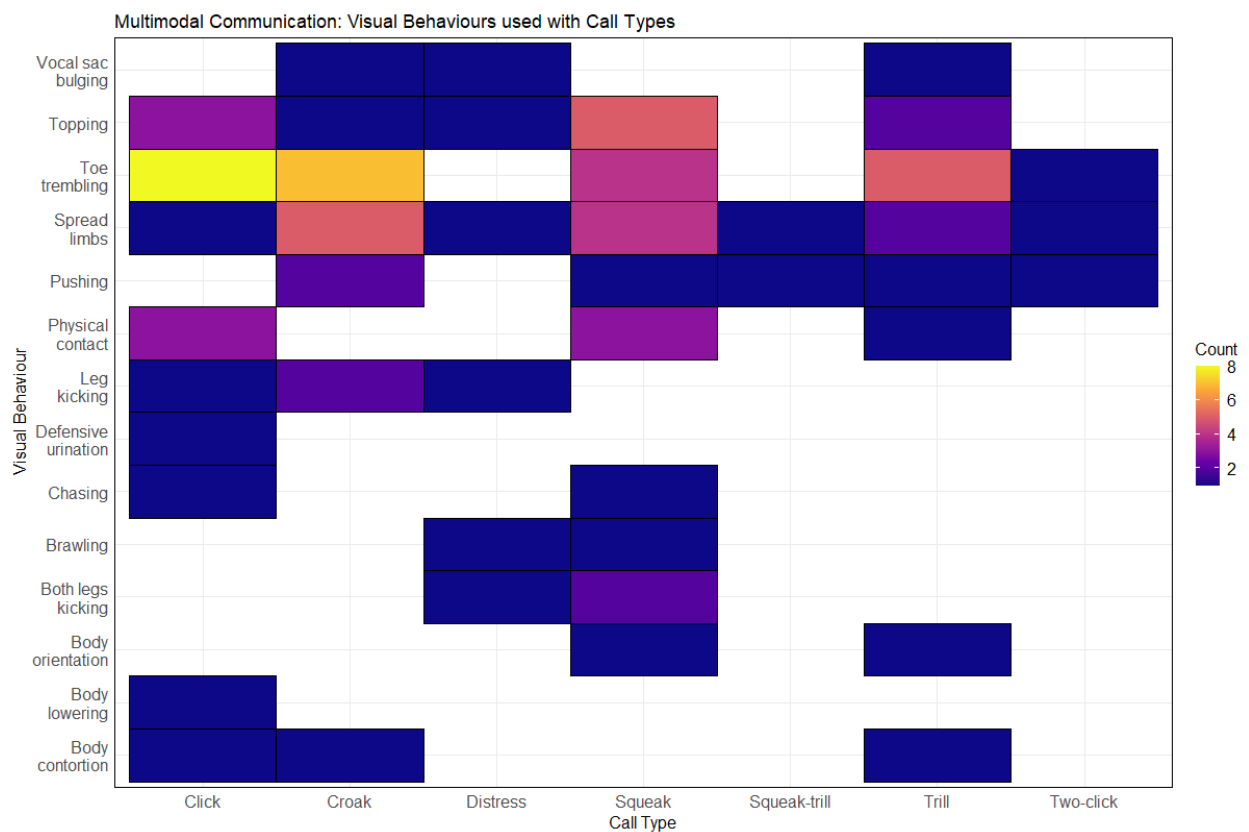


Figure 57. Tile plot illustrating visual behaviours in proportion to call types employed in multimodal communication. The legend indicates the counts of visual behaviours used with respective call types.

Behavioural contexts were identified for each call type employed in conjunction with visual behaviours and are summarised in Table 33. The number of individuals engaged in each individual multimodal interaction is summarised in Table 34. Most instances of multimodal interactions occurred between only two individuals. The substrate on which multimodal interactions were recorded are summarised in Table 35.

Table 33. Behavioural context identified for each call type employed in observed multimodal interactions.

Behavioural context of call type	n
Aggressive	74
Defensive	5
Reproductive	7
Total	86

Table 34. Number of individuals engaged in each recorded multimodal interaction, with the number of occurrences.

Number of individuals engaged in multimodal interaction	n
1	3
2	77
3	5
4	1
Total	86

Table 35. Substrate on which multimodal behaviours were recorded.

Substrate	n
Fern	36
Palm	23
<i>Scadoxus</i>	7
Vine	20
Total	86

Fisher's exact tests were conducted on selected variables across all four categories of observed behaviours to compare the overall distribution of counts across variables. These tests were conducted to gain a broad understanding of the relationships between variables. For the visual behaviours category, visual behaviours were tested against behaviour category and also tested against substrate. A robust association was found between visual behaviour

and behaviour category, with a p-value of < 0.001 . The same result was evident for visual behaviour vs substrate ($p < 0.001$). For the dual visual behaviour category, both recorded visual behaviours were tested against one another, yielding a p-value of 0.423, suggesting that there was no strong evidence of an association between the two sets of visual behaviours that were recorded. For the calling behaviours category, call type was tested against behavioural category in which those calls were emitted, yielding a p-value of < 0.001 , indicating a strong association between call types and behavioural categories of those calls. Call type was also tested against substrate, producing a p-value of < 0.001 , indicating that call types were significantly associated with the substrates on which these calls are emitted. For the multimodal behaviour category, Fisher's exact test yielded a p-value of 0.410 when testing visual behaviour against call type, indicating that there was no significant association between these two variables.

Chapter 4: Discussion

4.1 Egg clump monitoring

4.1.1 Egg clump monitoring protocol

Monthly rainfall was a significant predictor of the number of egg clumps recorded over the 2013–2023 timeframe. Slightly negative coefficient estimates of these linear models indicate that increased rainfall may slightly reduce the number of recorded egg clumps per month. This could be attributed to the occurrence of stream flooding in periods of heavy rainfall, where streamside vegetation becomes flattened and potentially washes some egg clumps away. Another plausible explanation may be that the rainfall data were collected on a monthly scale on a property that is nearly 10 km from the sampling sites, possibly introducing inaccurate estimates from statistical analyses. However, general trends from statistical analyses can still be recognised within and outside the *N. bonebergi* breeding season coinciding with climatic conditions. Monthly rainfall seems to influence the choice of oviposition sites in the streams, with more egg clumps found on attachment surfaces overhanging deeper water depths when decreased rainfall was recorded in a month. The same was also found for the inverse, where more egg clumps were found in shallower stream sections when increased rainfall was evident in a month. This can possibly be attributed to the spatial cognition capabilities of *N. bonebergi*, where oviposition sites at deeper, slow-flowing pools, with a higher likelihood of tadpole hatchling survival, may be chosen in drier months. Spatial cognition in amphibians is poorly understood because of the wide diversity of reproductive modes and behaviours which result in extreme diversity in their spatial ecology; however, spatial learning and flexible use of spatial memory are suggested to be utilised by dendrobatid frogs (Pašukonis et al. 2014). Therefore, further investigation into the spatial cognition capabilities of *N. bonebergi* may be warranted.

Kok and Seaman (1989) found that *N. bonebergi* lay their egg clumps at a mean height of 58 cm (SD = 34.1 cm) above water, however results from the EWT-TAP egg clump monitoring data show that the mean height of egg clumps for the 2013–2017 period was 67.2 cm (SD = 40 cm), and 77.7 cm (SD = 47.2 cm) for the 2018–2023 period - indicating that *N. bonebergi* egg clumps found in VCNR have been laid at greater heights over time. This may be attributed to the sensitivity or exposure of *N. bonebergi* in reaction to climate change over time (Dickinson et al. 2014). Numerous amphibian species were found to be affected by climate change, specifically extremes in weather such as drought, floods and storms (Blaustein et al. 2010; Combes et al. 2018; IUCN SSC Amphibian Specialist Group 2024). In southern

KwaZulu-Natal Province, a significant increasing trend in annual maximum rainfall occurrences has been found from climatological data from 1976–2016 at the Sezela Illovo Sugar Estate weather station, which is approximately 17.5 km from the VCNR transect site (Johnson et al. 2025). Although this weather station is coastal, the detection of increasing trends in rainfall maxima over a period of 40 years may still be applicable to VCNR. Flooding of streams in VCNR may therefore be an important factor in explaining why *N. bonebergi* lay their eggs at greater heights over time. Further investigation into the sensitivity or exposure risk of *N. bonebergi* in reaction to climate change would greatly improve conservation management of this species and its habitats in the face of a changing climate. April of the 2013–2017 period has the largest variation in the heights at which egg clumps were recorded, which is also evident for the month of March in the 2018–2023 period. This gives an indication of how spatial use of oviposition sites increase in variation at the peak of the *N. bonebergi* breeding season and how these variations in spatial use may have shifted to earlier months over time (Carey and Alexander 2003). However, this may also be due to other factors such as the availability of specific vegetation communities (i.e. oviposition sites) (Burrow and Maerz 2022), or changes in vegetation structure around streams throughout the sampling period and the *N. bonebergi* breeding season. It may also be related to increased movement and grazing by cattle within the reserve in more recent years.

Vegetation, specifically percentage cover, height stratification and community composition, can be a significant predictor of abundance in amphibians (Santi et al. 2010; Silva et al. 2012; Burrow and Maerz 2022), corroborating the finding that leaves were the most used attachment surface across the entire 2013–2023 data collection period. Coinciding with this, elevated monthly rainfall seemed to significantly influence the occurrence of egg clumps recorded on leaves and rocks during the 2013–2017 timeframe, but not in the 2018–2023 timeframe. Rock surfaces were recorded to be used as oviposition sites outside of the peak breeding season, which may indicate that spatial use of oviposition sites possibly shift to more suitable microclimatic conditions in the dry season. However, no measurements were taken to validate this speculation and future research on changes in microclimatic conditions throughout the *N. bonebergi* breeding season may provide clearer insights. This may extend to measuring microclimatic variations at different heights and levels of shade under which egg clumps were recorded. Most egg clumps were recorded under full shade during the total sampling period; however, a significant and positive relationship was found between egg clumps recorded in partial shade in the 2013–2017 period, indicating that oviposition is not exclusively restricted to fully shaded areas and that the microclimatic thresholds for oviposition sites may be flexible to some measurable degree. Furthermore, monthly rainfall had significant effects on the level of shade under which egg clumps were laid. For the 2013–2017 period, increased monthly

rainfall influenced *N. bonebergi* to lay slightly less egg clumps in “full sun”, “light shade” and “partial shade”, which may indicate that increased rainfall may have adverse effects on egg clumps on surfaces more exposed to direct rainfall. However, more egg clumps were laid in exposed positions (“full sun”) in the 2018–2023 period with increased monthly rainfall. These results are conflicting, but this may be attributed to the nature of the rainfall data being collected some distance from the transect sites. The increased monthly rainfall may have created more cloud cover over the VCNR transect sites during the 2018-2023 period, leading *N. bonebergi* to select these more exposed positions for oviposition sites. These findings may also be linked to increased cattle activity as a result of community incursions, and a decrease in availability and diversity of egg-attachment plants.

The number of eggs in egg clumps was found to have a strong correlation to the height at which they were laid above water for the entire dataset, where egg clumps containing more eggs were laid higher up and where water depth was shallower. This could be due to variation in oviposition site availability throughout the breeding season. This may link to other findings that egg clumps were laid closer to the water surface where water depth increased. These trends may be attributed to the stream- and vegetation composition throughout the VCNR transects, where deeper pools are often interlaced throughout the shallower stream sections (Kok and Seaman 1989). These deeper pools are often popular breeding sites for *N. bonebergi* in VCNR, perhaps because of ideal microclimatic conditions and vegetation structures around the pools, which would also be important for tadpole development. Since more egg clumps are generally found around these pools, it may influence the availability of ideal oviposition sites, compelling some females to select sites at shallower parts of the streams that are higher up. Hence, more information is needed on the breeding timing of females and oviposition site selection relative to site availability throughout the breeding season.

4.1.2 Egg clump isolation experiments

Clear differences can be observed in egg survivability between isolated egg clumps and controls for the February 2024 experiment. In the absence of female parental care, egg clumps are prone to desiccation and predation, even when most measurable variables were similar between the two groups. The April 2024 experiment showed fewer clear-cut differences in egg survivability between the three test groups. Desiccation may still occur in the absence of female parental care even when isolated egg clumps are artificially moistened. Isolated egg clumps for this experiment also only had one egg clump which desiccated when no artificial moistening was applied. Female parental care is thus essential to egg survivability, since no egg clumps desiccated in both control groups for each experiment. Parental care in *Eleutherodactylus*

coqui suggests a continuous transfer of water into eggs through physical contact of the individual's integument against the egg surface, thereby facilitating an increase in egg mass which consistently correlates with increased hatchling size and prevention of egg desiccation (Taigen et al. 1984). This may highlight the importance of females in making physical contact with eggs when moistening occurs, effectively transferring water via physical contact or voiding the bladder onto eggs, facilitating hydration. The difference in water potential between the female integument and the perivitelline space surrounding eggs likely facilitates the water uptake ability of eggs (Taigen et al. 1984). Simply dripping water onto eggs was therefore not sufficient to account for the water uptake ability of eggs to effectively prevent desiccation in the one egg clump in the moistening treatment. Temperature and relative humidity played important roles in Gosner Stage progression for all groups in the April 2024 experiment, indicating that the microclimates in which eggs are laid are also essential to their survival and development. Predation by *Crematogaster* spp. ants were evident in all isolation treatments, likely due to decreased jelly mass around embryos which makes it less difficult for insect mandibles to reach embryos when eggs are in a desiccated state (Touchon and Warkentin 2009). Additionally, more scent cues may be produced by desiccated eggs in comparison with hydrated eggs, which may alter predator foraging behaviour in the sheltered structure of streamside habitats in VCNR (Touchon and Warkentin 2009). Developmental rates of eggs differed for each group within each experiment. This could be due to egg clumps being isolated and monitored at various starting Gosner Stages. All egg clumps at early developmental stages show an increased growth rate which decreases over time, which is typical of embryological development being non-linear. At later stages of development, isolated egg clumps show slower rates of development when compared with controls. The egg clump moistening group shows rapid initial development when compared with isolated and control groups; however, this group contains less data points than the other two groups, which makes comparing the developmental rates between this group and the two other groups difficult.

4.2 Acoustic monitoring

4.2.1 Call profiles

Seven distinct call types were identified to be used by *N. bonebergi* throughout the breeding season, and these calls were divided into three functional categories as proposed by Toledo et al. (2015) and Köhler et al. (2017): reproductive, aggressive and defensive. Acoustic analyses of high-quality recordings obtained from the Zoom F1 directional microphone in this study indicate that *N. bonebergi* has a much more diverse acoustic repertoire than previously suggested by Wager (1965), Du Preez and Carruthers (2017) and Channing (2001). The

behavioural functions of these call types are mainly associated with male territoriality, with all call types being utilised in agonistic interactions between males to varying degrees of intensity. The “click” call type is the most frequently heard call from males on elevated perches. This call is also utilised independently from interactions with conspecifics, which corroborates known literature that this call serves both purposes of mate attraction and conveyance of territorial information to conspecifics, confirming the “click” call type as the advertisement call of the species (Köhler et al. 2017). This call type can be compared with the short, hollow tapping sound at regular intervals characteristic of the advertisement call of *Strongylopus grayii* (Du Preez and Carruthers 2017). The aggressive calls used by *N. bonebergi* males are employed relative to the intensity of agonistic interactions. These calls also vary widely in length and volume in any given interaction, and are likely also dependent on the relative intensity of the agonistic interactions these calls are employed in. The “croak” and “two-click” call types serve as encounter and warning calls in the “defensive calls” functional category, which are employed in most agonistic interactions before escalation occurs and males commence physical attacks on one-another. The “squeak”, “trill” and “squeak-trill” call types may also be employed in this behavioural context. These three call types are also utilised when interactions escalate in intensity and physical attacks commence. When agonistic interactions reach a terminus, the “distress call” and “squeak” call types are most often heard amongst brawling males. The “trill” call type can be compared with the advertisement call of *Strongylopus fasciatus*, as both calls share high-pitched chirp-like notes emitted consecutively at fast intervals (Du Preez and Carruthers 2017). Most *N. bonebergi* call types belong to the tonal sound category, as these calls contain a single frequency component at any given point in the call even as frequency and amplitude vary over time. The “squeak”, “squeak-trill”, “croak” and “distress call” call types can be categorised as complex and pulsatile, as fast alternating amplitude modulation is evident over the duration of each call. The “trill” call type can be described as pulsed because of clearly distinguishable amplitude modulations with silent intervals between notes. Categorising anuran call types serves to expand taxonomic knowledge and improve understanding of calling behaviour in social contexts (Köhler et al. 2017), as demonstrated in the case of *N. bonebergi*. Minima and maxima of call type dominant frequencies could be identified, and the differences evident between dominant frequency ranges of call types underscore the intraspecific call variation and diverse acoustic repertoire present in *N. bonebergi*.

4.2.2 Passive acoustic monitoring

The Raven Pro BLED Batch Detection tool proved to be a good tool to identify *N. bonebergi* vocalisations from passive acoustic monitoring data. Due to the intraspecific variability of calls and numerous distinctive call types of *N. bonebergi*, the BLED parameters had to be adjusted

to account for these variations. These broadened detection parameters coupled with a low score threshold produced numerous false positives in each output. This was an objective choice to minimise the presence of false negatives, thereby generating more accurate estimates of calling activity derived from the wide *N. bonebergi* repertoire. Even though some bird calls overlapped in detection parameters with *N. bonebergi* vocalisations, dominant frequencies of call types were still quite distinct from background sounds of other animals present at the SM4 site in VCNR. The bird calls were mostly detected during the mid-day when calling activity of *N. bonebergi* was low. Two distinct peaks in daily calling activity were evident: dusk to midnight and pre-dawn to early morning, indicating that *N. bonebergi* follows a clear circadian rhythm throughout the breeding season. When scaling calls to a monthly level, it was found that monthly rainfall has no clear influence on calling activity, but this estimate may be inaccurate as the rainfall data were collected some distance from the PAM site. However, when scaling calls to a daily level, mean daily temperature was shown to have a strong influence on daily calling activity. Large variation in recorded centre frequency (Hz) was observed to occur, coinciding with the *N. bonebergi* breeding season. A distinct rise of centre frequency occurs at the start of the breeding season and dips dramatically at the end of the breeding season. This may be attributed to energy expenditure and social contexts shifting throughout the breeding season, where males may expend more energy on calling at the heights of the breeding season when competition is at its fiercest (Köhler et al. 2017). These findings are consistent with anuran calling ecology being deeply connected with seasonality (Measey et al. 2017). These acoustic patterns of *N. bonebergi* are therefore found to be intimately tied to their environment and the social interactions present therein throughout the breeding season (Krause and Farina 2016). These findings contribute to expanding the body of knowledge on the various aspects which influence and parameterise environmental constraints, population dynamics, physiological rhythm and the acoustic phenology of *N. bonebergi* (Krause and Farina 2016; Desjonquères et al. 2020). Further studies may delve into PAM implementation at other altitudes and habitats in which *N. bonebergi* occur to gather more information on local spatial environmental effects on this species' acoustic phenology.

4.3 Observations of behaviour

4.3.1 Visual behaviours

Observations of visual behaviours exhibited by *N. bonebergi* were categorised according to the social contexts in which they were employed. A short communication article describing

these behavioural observations, parental care and predation of *N. bonebergi* was published in the African Journal of Ecology and attached in Annexure A (Van Niekerk et al. 2025). *Natalobatrachus bonebergi* males possess a complex and dynamic repertoire of visual and acoustic signals utilised in interactions between conspecifics, indicating that this species is not restricted to one mode of communication (Van Niekerk et al. 2025). Dense forested environments may provide the platform from which complex visual and acoustic modes of communication develop and evolve due to sound attenuation being relatively high in these environments, which is possibly the cause of the complex repertoire of *N. bonebergi*, evident in VCNR (Hödl and Amézquita 2001; Hartmann et al. 2005; Van Niekerk et al. 2025).

Males exhibited most of the aggressive and defensive behaviours associated with territoriality, as territorial defence of an area or resource is evident in a wide array of animal taxa and plays an important role in driving intersexual selection by females in *N. bonebergi* (Luría-Manzano et al. 2023; Van Niekerk et al. 2025). Reproductive behaviours recorded for males in relation to reproduction are limited to amplexus and egg fertilization. No behaviours were recorded to suggest that males employ courtship rituals toward females prior to amplexus attempts. Visual behaviours recorded for females are mostly attributed to parental care or reproduction. Males often mistake females swollen with water while tending to egg clumps for gravid females and in these cases, females make use of aggressive or defensive behaviours (i.e. leg kicking, head tilting, topping, pushing) to ward off such males. Head tilting in females was mainly used to forcibly remove males from the backs of females when amplexus was not warranted (Van Niekerk et al. 2025). In *Colostethus trinitatis* and *Staurois guttatus*, head tilting (or “upright posture”, exposing the ventral side of the body) was used by females when defending territories or repelling intruders, usually females (Wells 1980; Grafe and Wanger 2007). However, in cases where female *N. bonebergi* employed the head tilting behaviour, females were in the process of tending egg clumps and not reproductively active. In rarer cases, females were observed to use their forearms to accurately “poke” the eyes of amplexant males when all other attempts of removal were unsuccessful (Van Niekerk et al. 2025). Toe trembling was the visual signal most utilised in interactions between conspecifics in both sexes across all behavioural categories. This may indicate that toe trembling functions as an alerting signal to attract the attention of the signal receiver before the main signal is emitted (Lindquist and Hetherington 1996; Hödl and Amézquita 2001; Caldart et al. 2014; Starnberger et al. 2014; Van Niekerk et al. 2025). However, toe trembling was also observed to be used by females while moistening egg clumps, which prompts the question of what other functions toe trembling may serve if it is used outside of interactions between conspecifics.

Some visual behaviours exhibited by females could not be attributed to a specific behavioural category, as these behaviours were employed while no other individuals were

present in their immediate vicinity. Mouth gaping was recorded twice over the study period, once while the female was in amplexus and the other while the female was perched on a leaf some distance away from water and other individuals. Mouth gaping may be a defensive or agonistic behaviour in response to a potential predator, which is recorded in 15 other frog species and is always accompanied by defensive vocalisations in these species (Hartmann et al. 2005; Toledo et al. 2011). Regarding the contexts in which the two observations were made of mouth gaping in females, it is unlikely that this behaviour can be attributed to a defensive or agonistic behaviour. This behaviour may likely serve a physiological function in thermoregulation, similar to the evaporative cooling behaviour of panting observed in reptiles (Flouris and Piantoni 2015). However, further investigations are required to substantiate this hypothesis. Limb lifting in females was also observed, where females would slowly alternate between lifting their legs directly upward from the substrate. This behaviour is generally recorded in foot-flagging frog species (e.g. *Micrixalus saxicola*, *Hylodes asper* and *H. dactylocinus*) which is employed by males in agonistic interactions (Haddad and Giarretta 1999; Narvaes and Rodrigues 2005; Preininger et al. 2013). Limb lifting was only observed in females facilitating egg clump moistening; evident from their abdomens swollen with water which is transported to egg clumps for moistening. This behaviour could also not be attributed to any single behavioural category, as no other individuals were present in the females' immediate vicinities. This behaviour was observed once where a female was exhibiting toe trembling while tending to an egg clump. Other behaviours were employed with limb lifting in females with swollen abdomens, such as toe trembling and head tilting, where no other individuals were present. This visual behaviour may be linked to physiological factors in females facilitating parental care for egg clumps, as their abdomens swell distinctly while absorbing water and seem to exert great effort in transporting this liquid to egg clumps. Therefore, limb lifting and head tilting may serve as a mechanism to reduce the physiological stress that females experience while carrying large quantities of water to egg clumps. More information is needed to substantiate this hypothesis. Anti-predator defence responses were observed in *N. bonebergi* embryos prior to hatching, where mechanical disturbance of eggs induced mass-evacuation of some eggs at advanced stages of embryological development.

4.3.2 Multimodal behaviours

Profiled call types utilised in interactions between conspecifics were attributed to different behavioural contexts and subsequently categorised. Clicks were mostly used in reproductive contexts, but also across all other behavioural categories. The extensive use of clicks in various behavioural contexts could be attributed to this call also functioning as an alerting signal, effectively drawing attention to the emission of a second signal, which may be a visual or acoustic signal (Grafe and Wanger 2007; Preininger et al. 2013). Visual behaviours

and call types were significantly associated with the substrates on which these respective behaviours were recorded. Males typically identify suitable oviposition sites which are relatively unobscured by other vegetation and overhang water (Du Preez and Carruthers 2017). This may be attributed to the spatial use, oviposition site availability and territoriality of *N. bonebergi* throughout the breeding season (Hödl and Amézquita 2001; Pašukonis et al. 2014; Burrow and Maerz 2022). Multimodal communication in *N. bonebergi* was evident throughout the study period, as multiple behaviours were recorded to be used with various call types. Multimodal communication is defined as the combination of visual and acoustic signals to convey more complex signals to receivers (Grafe and Wanger 2007; Köhler et al. 2017). Multiple hypotheses have been proposed to explain the functions of multimodal communication, some postulating that individual signals in a multimodal signal convey a specific message for each signal (Johnstone 1996), while others posit that multiple signals are used to convey a single message (Moller and Pomiankowski 1993; Grafe and Wanger 2007). For the present study, no significant association of individual visual signals corresponding to specific call types were found, pointing to the plausibility of the multiple messages and efficacy-based hypotheses, which state that individual signals can convey different messages and multimodal signalling strategies are favoured where detection of signals may be hampered by attenuation and signal degradation in noisy environments (Johnstone 1996; Hödl and Amézquita 2001; Grafe and Wanger 2007).

4.4 Conclusions

Amphibians serve important roles as indicators of ecosystem integrity and environmental health. This group faces multiple threats causing the worst declines across any vertebrate group, demanding that more attention and conservation action is needed for this overlooked and under-resourced Class (Valencia-Aguilar et al. 2013; Campbell Grant et al. 2023; IUCN SSC Amphibian Specialist Group 2024). The Endangered *Natalobatrachus bonebergi* faces numerous threats across its range, including pollution associated with water quality, loss and fragmentation of habitat caused by agriculture and residential development, and harvesting of species associated with egg-laying (IUCN 2016). A monitoring and surveillance protocol was established in 2013 to help understand the impacts of threats on reproductive success (Tarrant and Armstrong, 2013). Rainfall could affect the breeding success of *N. bonebergi* in VCNR, as preliminary findings suggest that rainfall has some influence on the number of egg clumps found throughout the breeding season. Since the rainfall data was collected on a monthly basis some distance away from the egg clump monitoring sites, more accurate data is needed to confirm these preliminary findings. Vegetation communities within VCNR are an important factor influencing how this species utilises streamside vegetation to facilitate reproduction. These findings have implications for management of invasive vegetation and anthropogenic

disturbances across the range of this species. More information is needed on site fidelity and oviposition site selection by females, as shade and height above water might form only a part of the influences that determine oviposition site selection. Microclimatic changes, as well as resource use changes in the form of livestock, over time in VCNR may have caused this species to shift their breeding timing and heights at which they lay their eggs, prompting further investigation into the roles that climate change and livestock management has to play in these shifts. The latter also has implications for community engagement. Temperature and relative humidity are important factors in egg clump survival, however, the most important factor for egg survivability in *N. bonebergi* is parental care facilitated by females. If females are prevented from tending to egg clumps, embryo mortalities are far more likely to occur because of desiccation or predation. The developmental rate of embryos is influenced by parental care as well, however more information is needed on specific physiological changes throughout embryological development in relation to female parental care. Acoustic monitoring and observations on their reproductive behaviour have been facilitated for this species, shedding light on how this species interacts with conspecifics within their habitat over the course of their breeding season. *Natalobatrachus bonebergi* was found to have a complex acoustic and behavioural repertoire because of selective pressures imposed upon this species by fierce territoriality and their forested environment. This species may serve as an important ecological model by which more knowledge on communication and reproduction in amphibians may be gathered.

Natalobatrachus bonebergi is a remarkably unique species of frog, evident in its extensive and complex repertoire of communication and adaptable utilisation of their habitat in the face of climatic and structural changes over time. Reproductive behaviours investigated throughout this study provide mere glimpses into the intimate relationship this species shares with its environment and conspecifics.

Many hours of dedicated fieldwork and time spent observing *N. bonebergi* up close, have consistently reshaped how I think of amphibians interacting with their conspecifics and their environment – leaving me now in a state of awe at this group's complexities, which are always deeper than the eye can see.

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SHORT COMMUNICATION OPEN ACCESS

Kickboxing Kloof Frogs: Territoriality, Female Parental Care and Other Reproductive Behaviours of *Natalobatrachus bonebergi*

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ABSTRACT

The Kloof Frog (*Natalobatrachus bonebergi*) is an Endangered South African endemic, demonstrating complex reproductive behaviours. This study, conducted in Vernon Crookes Nature Reserve, South Africa between April 2023 and April 2024, recorded acoustic and visual communication, male–male aggression, parental care and predation. In situ observations underscore the species' strategies of multimodal communication in male territoriality, mate attraction and female parental care of eggs in a structured habitat. This study provides perspective on the reproductive ecology of *N. bonebergi*. This species could serve as an important ecological model through which conservation management and actions could be improved upon.

1 | Introduction

Territorial behaviour is not uncommon among amphibians and fierce territorial behaviour has been documented for *Pyxicephalus adspersus* (see Kok, Du Preez, and Channing 1989; Engelbrecht, Mashao, and Halajian 2015; Du Preez and Carruthers 2017), *P. beytelli* (see Du Preez et al. 2024) and *P. edulis* (see Engelbrecht, Mashao, and Halajian 2015). Among male Grey Tree Frogs (*Chiromantis xerampelina*) males have been reported to compete for the chance to mate with a spawning female (Jennions, Bakwell, and Passmore 1992).

The absence of a protective shell around amphibian eggs makes them vulnerable to numerous threats, such as desiccation, predation and bacterial or fungal infections. Parental care in amphibians has independently evolved across various amphibian

families (Wells 2007) and include egg attendance, egg transportation, tadpole attendance, tadpole feeding, as well as the transportation of tadpoles and metamorphs. In southern Africa parental care has been documented for *Pyxicephalus adspersus* (see Kok, Du Preez, and Channing 1989) and *Hemisus marmoratus* and *H. guttatus* (see Kyle and Du Preez 2020).

The Kloof Frog (*Natalobatrachus bonebergi* Hewitt and Methuen, 1913) is a cryptic and Endangered species endemic to coastal forest regions of KwaZulu-Natal Province and the Eastern Cape Province, South Africa (Du Preez and Carruthers 2017; IUCN 2016; Measey (ed.) 2011; Minter et al. 2004). *Natalobatrachus bonebergi* is a semi-arboreal riparian forest specialist, inhabiting densely forested ravines with rocky streams (Kok and Seaman 1989; McLean et al. 2016; Minter et al. 2004). In Vernon Crookes Nature

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Reserve on the KwaZulu-Natal south coast, *N. bonebergi* occurs in streams with low banks that are shallow (30–40 cm) and relatively narrow (1–2 m) (Kok and Seaman 1989). These streams have short sections of fast-flowing water, alternating with larger sections of slow-flowing water and pools which vary in depth and size. A variety of vegetation overhangs the streams at various heights, consisting of trees, shrubs, vines, forbs, grasses and sedges and rocks of various sizes line the banks. Reproductively active frogs are not found where the streams are wider and deeper, nor in somewhat marshy or open regions (Kok and Seaman 1989). This monotypic species has developed complex behavioural and reproductive mechanisms crucial to survival in this highly structured and sheltered habitat. The extended breeding season of *N. bonebergi* occurs mostly from October to May, where breeding occurs continuously during the rainy summer months (Kok and Seaman 1988, 1989). *Natalobatrachus bonebergi* performs reproductive behaviour nocturnally (Kok and Seaman 1989).

In the majority of anurans, acoustic communication is essential for reproductive success and sexual selection (Narins et al. 2018; Starnberger, Preininger, and Hödl 2014). The species' advertisement call of *N. bonebergi* has been described as an inconspicuous click or "chick", akin to water dripping slowly at irregular intervals (Channing 2019; Wager 1965). During the breeding season, males select a perch above the water surface from which they emit advertisement calls to attract females and possibly establish and defend territories of sites suitable for oviposition (Du Preez and Carruthers 2017; Kok and Seaman 1989; Narins, Hödl, and Grabul 2003; Starnberger, Preininger, and Hödl 2014). Female *N. bonebergi* lay their eggs in gelatinous masses of 75–108 eggs at heights from about 0.2 to +2.0 m above the water surface (mean height ~ 58 cm) (Channing 2019; Kok and Seaman 1988). Females lay only one egg clutch at a time, the female tends to the clutch by moistening the eggs with urine until the tadpoles are near hatching, and after approximately 4–6 days the tadpoles drop into the water below (Du Preez and Carruthers 2017; Wager 1952). The unique breeding biology and phenology of this monotypic species are incompletely known. The forest habitat of *N. bonebergi* are threatened by agriculture, especially sugarcane cultivation, afforestation, woodcutting and urbanisation in the KwaZulu-Natal Province (Du Preez and Carruthers 2017; IUCN 2016; Tarrant and Armstrong 2013). Mining activity, pollution and siltation are also threats this species experiences (IUCN 2016). Although this species has an extended breeding season from October to May (Kok and Seaman 1989), little knowledge has been gathered on the specific reproductive behaviours these frogs exhibit, such as acoustic patterns, parental care, oviposition, site selection and male competition. Observations are lacking about the social contexts in which *N. bonebergi* expresses reproductive, aggressive or defensive behaviours. For this study, field observations were conducted for 8–14 days every 3 months at Vernon Crookes Nature Reserve (VCNR), KwaZulu-Natal, between April 2023 and April 2024. Vocalisations are the primary mode of inter- and intrasexual communication in anuran amphibians; however if successful strategies of communication in noisy environments are beneficial for both sender and receiver, communication strategies in both the acoustic and visual domain can develop (Starnberger, Preininger, and Hödl 2014). Knowledge

of a species' reproduction and ecology contributes to the assessment of the species' conservation status and is necessary for the development of conservation strategies and action plans using established IUCN criteria (Hoffmann et al. 2008; Noronha and Rodrigues 2018). The objective of this study is to document and quantify reproductive behaviours of *N. bonebergi* in relation to conspecifics and environmental context. The following behaviours were documented during the study period.

2 | Observations

2.1 | Methodology

The study was conducted at Vernon Crookes Nature Reserve (−30.30996 30.54687, −30.25593 30.62644), with a minimum elevation of 118 m and maximum elevation of 588 m. The nature reserve is 2189 ha in size, encompassing extensive coastal grasslands and coastal forest habitats including forested ravines interlaced with networks of slow-flowing streams. All field observations were made primarily by one observer, using a combination of photographs, video recordings and written notes on observed behaviours. Field observations commenced on 11 April 2023 for 4 days; for 4 days in June/July 2023 to observe behaviours outside *N. bonebergi* breeding season; then every 3 months for 8–14 days and concluded on 22 April 2024 with 10 days of field observations. Additional observers were occasionally present at the study site to facilitate video recordings and written notes of behaviours. Effects of disturbance on *N. bonebergi* behaviours through observer presence were minimised by recording behaviours from distances of at least 60 cm. Observations were started 2 min after approaching individuals engaged in interactions to allow normal behavioural activity to resume. Behavioural observations were usually made daily at one popular breeding site in VCNR from approximately 19:00 to 23:00.

2.2 | Male–Male Aggression

Fierce competition was observed to occur between male conspecifics. In addition to the males' extensive acoustic repertoire, numerous visual behaviours are used in agonistic interactions between male conspecifics [Table 1]. All of the listed behaviours in Table 1 were quantified from video and written material taken in situ; however, some territorial agonistic behaviours (brawling, chasing, bumping, pushing, body orientation, leg kicking and topping) were mostly observed incidentally, as these behaviours occurred quickly and at instances when recording was not feasible—these instances were not quantified in Table 1. Males with established territories become highly defensive during the breeding season, enforcing boundaries with great vigour against intruding males. This activity is not evident outside of the breeding season. In most instances, intruding males are silent, and lower their bodies and spread their limbs over the substrate (usually a leaf, branch or rock), presumably to either signal lack of aggression or submission when detected by the defending male. Sometimes intruder males attempt to challenge and defeat the defending male, thereby claiming the calling territory

TABLE 1 | Behaviours employed by Kloof Frog males and females in agonistic interactions. Frequency of behaviour is the number of times that a behaviour was observed during the study period on video camera (136 total minutes) and 12 notebook entries.

Behaviours	Sex	Context	Frequency of behaviour (<i>n</i>)
Toe trembling	M, F ^a	Alerting signal/ritualised behaviour	108
One leg kicking	M	Terminal territorial agonistic	41
Topping	M	Low-intensity territorial agonistic	30
Spread limbs	M	Low-intensity territorial agonistic/low-intensity defensive	24
Both legs kicking	M	Terminal territorial agonistic	17
Physical contact	M	Low-intensity territorial agonistic	16
Pushing	M, F	Low-intensity territorial agonistic	15
Body orientation	M	Low-intensity territorial agonistic	9
Vocal sac bulging	M	Low-intensity territorial agonistic	5
Body contortion	M, F	Low-intensity defensive/amplexus release signal	5
Chasing	M	High-intensity territorial agonistic	5
Defensive urination	M	Terminal defensive	4
Body lowering	M	Low-intensity defensive	4
Bumping	M	High-intensity territorial agonistic	2
Brawling	M	Terminal territorial agonistic	1

^aToe trembling by females is not necessarily agonistic; it is also employed in reproduction and parental care.

as their own. In both scenarios, agonistic interactions ensue. Defending males use an array of acoustic and visual signals in these closer-range interactions. These males employ combinations of clicks, trills, croaks and squeaks with visual and physical behaviours. These behaviours include climbing on top of intruders (topping) while calling (Figure 1a), toe trembling, body orientation, physical contact, and rarely, vocal sac bulging to intimidate and ward off intruders. Toe trembling is employed directly before several agonistic, defensive or reproductive behaviours. Males occasionally tremble their toes inbetween calling when defending their territories, but mostly in agonistic interactions. When none of these combinations are successful in deterring intruder males from an established territory, the defending males resort to kicking and brawling. Defending males position themselves in front of intruders, orienting their bodies to face away from intruders and lining themselves up for powerful kicks. With great accuracy, defender males kick at the face of intruders, usually sending intruding males flying from the calling sites (Figure 1b). When kicking does not remove or deter intruders, explosive brawling ensues with a cacophony of high-pitch squeaks, kicks and tumbles until one or both males fall from the elevated calling territory.

2.3 | Amplexus and Spawning

Gravid females approach calling males situated on suitable surfaces, such as leaves, branches or rocks, when they are ready to lay their eggs. No visual or acoustic signals indicative of courtship are given by either males or females prior to amplexus. Amplexus is axillary and lasts anything from 30 min to 6 h and typically occurs at night (Figure 2c). However, on 9 November 2023 at 12:13 pm a pair was seen in amplexus. Egg laying takes approximately 10 min. External fertilisation occurs as the female contracts her abdomen to release eggs and the male responds by contracting his abdomen immediately after the female does to release sperm. Directly prior to the female contracting her abdomen to release eggs, both male and female tremble their toes. Females then rub their legs, particularly their thighs, over the eggs. Males disengage from amplexus and move away once all eggs are laid. The females stay and continuously rub their legs and thighs over their eggs. Once, two satellite males were observed quickly approaching a female, 2 min after all her eggs were laid and the initial male had moved away. One of the satellite males succeeded in initiating amplexus, releasing sperm on the already laid eggs from the back of the female while she continuously rubbed her eggs (18 April 2024).

Numerous behaviours are employed by unreceptive females when amplexus is not warranted, usually initiated by males when females are tending to their laid egg clumps. Males are often confused by females with swollen abdomens, mistaking females transporting liquid to moisten their egg clumps for females that are gravid. When males attempt amplexus with unreceptive females, the females will tilt their heads back up to approximately 80° to push the males from their backs (Figure 2d), or the females may contort their abdomens to loosen the grip of the male's forelegs. In other instances that have been recorded on video camera, unreceptive females will accurately kick males away when amplexus is attempted. In rarer cases,

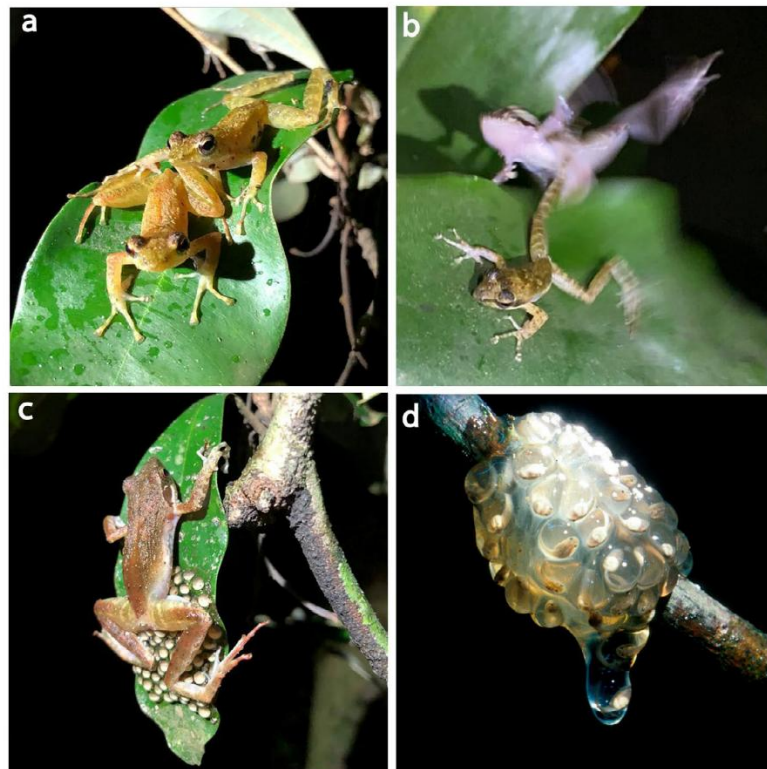


FIGURE 1 | (a) Two male *Natalobatrachus bonebergi* in an agonistic interaction. (b) Male *N. bonebergi* kicking an amplexant pair from his territory. (c) Female tending to her egg clump. (d) Hatching *N. bonebergi* tadpoles of an egg clump attached to a twig.

females use their forelegs to quickly reach back and very accurately “poke” the eyes of the amplexant males to make the males release them.

2.4 | Parental Care

Natalobatrachus bonebergi females express high levels of parental care for their egg clutches up until the late embryological developmental phase of the tadpoles. After hydrating themselves in the stream, females make their way to individual egg clutches, depositing the liquid from their swollen bladder in small amounts onto eggs while dispensing the liquid all over the eggs with their legs. Females grip the egg-attachment surface firmly with their hands while rubbing the liquid thoroughly over the egg clutch using their hindlegs and feet (Figure 1c). Some egg clutches that have been abandoned by females become infected by unidentified fungi ($n=13$). However, whether the females abandon the egg clutches before or after the onset of fungal infestation, or whether the female stopped tending to their egg clutches due to other reasons (e.g., death), has yet to be established. Parental care in neotropical glass frogs (*Teratohyla pulverata* and *Hyalinobatrachium cappellei*) reduces embryo mortality from fungal infection, dehydration and predation, especially within the first 2 days after oviposition (Delia, Bravo-Valencia, and Warkentin 2017; Noronha and Rodrigues 2018). Females regularly visit and moisten their egg clutches immediately after laying, with the visit frequency being highest during

the early phases of embryological development. With embryonic growth, the jelly mass surrounding each egg increases in size, gradually reducing the need for females to protect them from desiccation. Once the embryos reach a developmental stage where additional moisture from the female is no longer necessary (around Gosner Stage 18), females stop visiting the clutch entirely (Figure 1d). Tadpoles were observed to take at least 8 days to commence hatching throughout the study period.

2.5 | Predation

Several instances of predation on *N. bonebergi* were observed. Ants (*Crematogaster* sp. and an unidentified species) feed on egg clumps which have been abandoned by females or egg clumps that have begun desiccating, completely devouring the gelatinous parts of these egg clumps within approximately 24 h (14 observed cases during February and April 2024). The *Crematogaster* sp. is relatively large, with a red head and thorax and a large abdomen that is orange with a pointed black tip. The unidentified ant species is small and pale yellow. *Crematogaster* sp. ants consume egg clumps while the egg clumps are mostly moist (Figure 2a), while the unidentified species usually starts feeding on the egg clumps when they are mostly desiccated.

Brown water snakes (*Lycodonormorphus rufulus*) make extensive use of the stream vegetation to conceal their ambush efforts, occupying ferns, palms and surrounding trees, from water level to

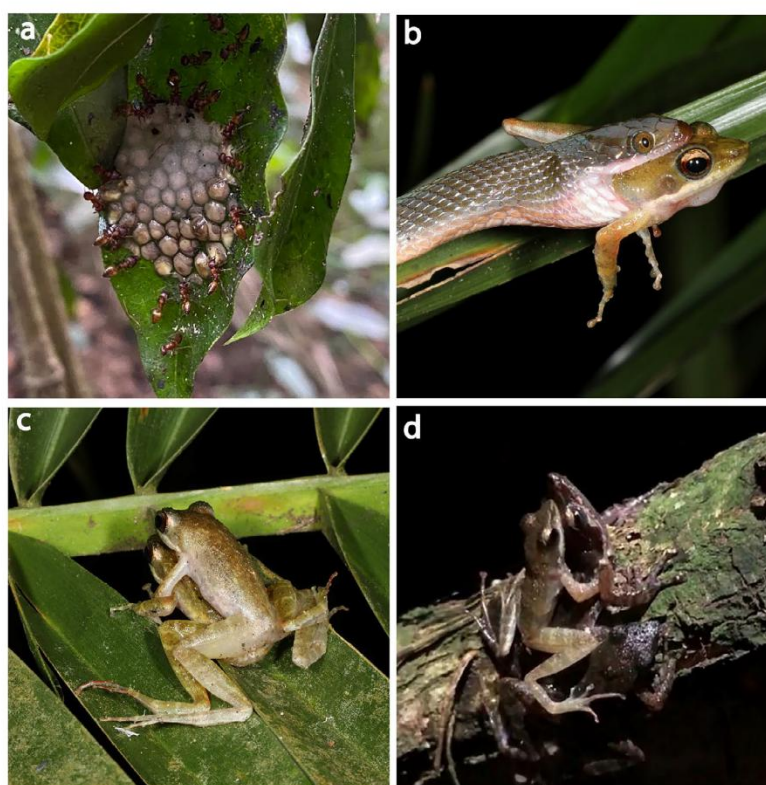


FIGURE 2 | (a) *Crematogaster* sp. ants consuming an abandoned and partly desiccated egg clump. (b) *Lycodonomorphus rufulus* ingesting a male *Natalobatrachus bonebergi*. (c) Amplexant pair. (d) Male *N. bonebergi* attempting amplexus on an unreceptive female (female tilts head approximately 80° to cause male to release).

3 m up in tree canopies. Three predation events were recorded as follows. On 12 April 2023, an adult *L. rufulus* slowly advanced up a palm rachis to four male *N. bonebergi* which were situated on the terminal leaflets of the palm, seizing two males in a span of approximately 20 min (Figure 2b). On 8 February 2024, a juvenile *L. rufulus* used the same tactic on another palm on the opposite streambank where five male *N. bonebergi* were situated on the terminal leaflets. The juvenile snake was too short to reach the frogs that were clinging vertically to the leaflets, so the snake coiled its tail around the rachis of the palm and slowly edged closer to the frogs while poised to strike. In an instant, the snake released its grip on the rachis and slid down the leaf, catching the closest male frog before dropping into the stream below and retreating to a hole in the streambed under the palm [Video S1]. The frogs were consumed head first for two of the predation instances, and once legs first.

Another suspected predator of *N. bonebergi* adults is the rain spider (*Palystes castaneus*). In November 2023, a very large *P. castaneus* was found in ambush-hunting position approximately 1.5 m above the water surface, surrounded by seven apparently unsuspecting male *N. bonebergi* in its immediate vicinity. Twice, fishing spiders (*Nilus* sp.) were found biting egg clumps on which they rested, most likely due to felt movements from the tadpoles inside. Numerous *Nilus* spiders were observed in hunting posture with their front four limbs on the water surface, apparently

waiting to catch *N. bonebergi* tadpoles moving below them, as well as other water insects that were present.

A corpse of a male *N. bonebergi* was discovered in November 2023 with its eyes and stomach contents removed, and from the frog's injuries, a river crab (*Potamonautus* sp.) was suspected to be the predator. Another instance of a *Potamonautus* crab attempting to reach low-hanging egg clumps was also recorded in the observation period. The *Potamonautus* crab's fundamental niche overlaps with that of *N. bonebergi* around stream beds, making *Potamonautus* crabs suspected frequent predators of *N. bonebergi* and opportunistic feeders of low-hanging egg clumps.

In February 2024, a water scorpion (Family Nepidae) grabbed hold of a leg of a male *N. bonebergi* that fell from an elevated calling site into a shallow part of a pool occupied by numerous water scorpions, and did not release it until the frog managed to escape after struggling for approximately 1 min. On occasion, unidentified insect larvae were observed on the egg clumps, but were never seen penetrating the eggs.

3 | Discussion

Visual displays from male *N. bonebergi* were observed to be presented independently from vocalisations during agonistic

interactions between males in the study period, indicating that this species has a much broader signal repertoire than what was initially thought. It has become evident that *N. bonebergi* does not limit communication between conspecifics to one mode of communication (i.e., acoustic communication), but rather possesses a complex repertoire of acoustic and visual signals which are used simultaneously or sequentially in interactions with conspecifics at monitored breeding sites within VCNR. Sound attenuation is relatively high in dense forests, providing the grounds for the development and evolution of complex acoustic and visual behavioural repertoires (Hartmann et al. 2005; Hödl and Amézquita 2001). The streams flowing through the dense scarp and gallery forests which *N. bonebergi* inhabit could serve as ample grounds from which this species may have developed its suite of visual behaviours to facilitate the sending and receiving of signals in a noisy environment. Multiple additional call types have been observed to be utilised throughout the breeding season, most of which being employed in agonistic interactions between male conspecifics. Visual behaviours were also observed to be employed in conjunction with these different call types in some agonistic interactions, ranging from visual signalling to physical brawling. Whether females choose suitable oviposition sites, suitable males, or vice versa remains unknown. Territorial defence of an area or resource involved in reproduction is evident across animal taxa and is facilitated through diverse displays of behaviours, usually through a graded system from little aggressive non-physical displays through to very aggressive physical disputes (Luría-Manzano et al. 2023). This is also evident in male *N. bonebergi*, where males establish and defend areas or sites suitable for oviposition. These sites are potentially characterised by the suitability and stability of the attachment surface for egg clutches and position and height above the water level. Territoriality may promote intrasexual selection through the increase of male individual fitness via competition between conspecific males as well as intersexual selection through the selection of males by females (Luría-Manzano et al. 2023). For *N. bonebergi*, territoriality may be an important driver of intersexual selection. Some males were observed to defend exposed sites more vigorously than sites sheltered by other vegetation. This might be done to increase attractiveness and site visibility to females; however more research is needed to validate this. This behaviour may also have developed due to the selective pressure of a noisy environment, where visual signalling of individual fitness may have contributed to the evolution of the trait, in addition to territoriality (Hödl and Amézquita 2001). Visual signals may also function as alerting signals, for instance, toe trembling may be employed as an alerting signal to attract the attention of the receiver before the main signal is emitted (Caldart, Iop, and Cechin 2014; Hödl and Amézquita 2001; Lindquist and Hetherington 1996; Starnberger, Preininger, and Hödl 2014). This could potentially explain the extensive use of toe trembling in many interactions with conspecifics in *N. bonebergi*; however more information is needed to justify this. Some *N. bonebergi* males which intrude onto the territories of other males will lower their bodies against the substrate, which is a typical behaviour in anurans to signal lack of aggression or submission (Hödl and Amézquita 2001). Knowledge about anuran communication is increasing rapidly (Preininger, Boeckle, et al. 2013; Starnberger, Preininger, and Hödl 2014). Multimodal communication has been found in numerous families and genera of anurans worldwide, showing that anurans are exemplary model

organisms for studying alternative or multimodal communication and how these communication methods have evolved (Noronha and Rodrigues 2018; Preininger, Boeckle, et al. 2013; Starnberger, Preininger, and Hödl 2014). Numerous strategies of communication and territoriality are employed by male *N. bonebergi* to ensure reproductive success, accentuating this species' intricate relationships with conspecifics and their adaptation to a complex habitat. More research needs to be conducted on signal reception in these contexts. Mate selection criteria of females are not yet fully understood (Kok and Seaman 1989; Hödl and Amézquita 2001; Narins, Hödl, and Grabul 2003; Preininger, Stiegler, et al. 2013); however, male calling ability and territoriality may play a role.

The arboreal egg-laying behaviour of *N. bonebergi* may have evolved to increase the likelihood of clutch survival by protecting the eggs from aquatic predators (Touchon and Worley 2015) because *N. bonebergi* usually lay their eggs above water, ranging from 8 cm above the water surface to a maximum of 4 m in some observed cases (Kok and Seaman 1989). However, other threats emerge through desiccation and arboreal foragers or predators, such as ants, crickets and wasps (Noronha and Rodrigues 2018). To account for these risks, *N. bonebergi* females have developed parental care to increase the survival likelihood of offspring by preventing desiccation, predation and fungal attack, often at the cost of risking their own survival (Noronha and Rodrigues 2018; Royle, Smiseth, and Kölliker 2012). Females of other frog species that exhibit parental care, such as *Teratohyla pulverata* and *Cochranella granulosa*, spend significant time and energy protecting their newly laid eggs from desiccation and possibly predation (Delia, Bravo-Valencia, and Warkentin 2017; Salica, Vonesh, and Warkentin 2017; Lehtinen, Green, and Pringle 2014). Females absorb water through the skin of the posteroventral region, termed the pelvic patch, transporting the water to their own egg clumps to dispense onto and hydrate their eggs (Delia, Ramírez-Bautista, and Summers 2013; Taigen, Pough, and Stewart 1984). The majority of arboreal and terrestrial frogs have specialisations in the form of elevations and grooves in the skin, named verrucae hydrophilicae, for rapidly absorbing water in the pelvic patch region (Ogushi et al. 2010). *Natalobatrachus bonebergi* are semi-arboreal riparian forest specialists which may have adopted similar traits over evolutionary time. Female *N. bonebergi* could have these specialisations which may also function to hydrate eggs; however further research needs to be conducted to validate this. Females rub their legs over the entirety of the egg clump while liquid is excreted from the ventral integument or cloaca; this behaviour may function to hydrate and protect the eggs from any potentially harmful pathogens or debris (Lehtinen, Green, and Pringle 2014; Noronha and Rodrigues 2018; Salica, Vonesh, and Warkentin 2017). It is unknown whether urine is the only substance being excreted onto egg clutches to facilitate moistening, and whether females definitively only tend to their own egg clutches.

The reproductive behaviour of *N. bonebergi* is multifaceted and complex, reflecting the evolutionary distinctness of this monotypic species and its dependence on its structured habitat. In situ observations have contributed significantly to our understanding of some of these behaviours, as they have never before been recorded for this species in any available database.

Knowing that these frogs only lay their eggs at specific heights above slow-flowing water and never in areas bereft of shade already provides an ample frame by which conservation management may be improved. Further research is necessary to deepen understanding of the sending and receiving of signals in the context of *N. bonebergi* reproduction and its environment. Factors influencing sites of oviposition also requires further investigation, as it is still unknown whether females select sites based on male fitness or site suitability or perhaps both. *Natalobatrachus bonebergi* may be a cryptic species, but may serve as an exemplary species to study avenues of reproductive behaviours, specifically: multimodal communication between males and females (acoustic, visual, seismic and chemical), territoriality, courtship, maternal care of eggs and site fidelity. Natural history studies form the foundation of scientific progress, and serve to improve conservation efforts for threatened taxa. Much knowledge and numerous insights have been gained on the fascinating phenology of this species. *Natalobatrachus bonebergi* may serve as an important ecological model in the study of behavioural and environmental factors that could increase reproductive success in arboreal egg-laying anurans.

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Ethics Statement

This study was approved by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) on 18/05/2023 (Ethics number: NWU-00767-23-A5). This study was also approved by Ezemvelo KZN Wildlife Permits Office on 05/04/2023 (Permit number: OP 1231/2023).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data used in this paper were captured in the form of videos and photographs by authors Arno van Niekerk and Louis H. du Preez. [Supporting Information](#) on behavioural observations were captured in notebooks in situ by author Arno van Niekerk.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.