First frost, last frost: Updating current knowledge on the seasonality of intermediate snail hosts of *Fasciola hepatica* in the Southern Tablelands of New South Wales

Abstract

Fasciola hepatica is a zoonotic flatworm of global concern. The parasite is dependent on temperatures >10°C and adequate moisture for several stages of larval development, presence, and infection of their intermediate lymnaeid snail hosts. Ruminant livestock producers in the Southern Tablelands of NSW leverage the seasonal pause in the life cycle between the first frost in May, and last frost in September, to guide their treatment schedule. Whilst conducting a drug resistance trial in July 2024, numerous active lymnaeid snails were discovered outside the historical dormancy window in the region when average daily temperatures were <7°C, challenging the foundations historic integrated parasite management (IPM) strategies are based upon. This study set out to update knowledge on the seasonality of lymnaeid snail populations in the region to help redefine current *F. hepatica* risk periods and inform new IPM strategies. To achieve this, twelve sites across six farms endemic for *F. hepatica* suspected to be favorable to lymnaeids were sampled bi-monthly for one year. Collected snails were counted and morphologically speciated to determine the average abundance and diversity of susceptible intermediate hosts. Snails were first screened for *F. hepatica* larval infection by visual inspection for larvae, then confirmed with quantitative polymerase chain reaction (qPCR). One site (F2) did not follow the historical seasonal abundance pattern, recording the highest abundance (N = 240) of lymnaeid snails in the study, and in July when the mean weekly temperature was <8°C. The invasive snail species *Pseudosuccinea columella* was also most abundant in July at three sites (A2, E2, and F2). These results highlight the urgent need for further investigation to confirm the infective risk of these invasive intermediate hosts during the historical dormancy window.

Keywords

Lymnaeid Snail, Seasonality, Invasive Species, Integrated Parasite Management, Liver fluke

1. Introduction

Fasciola hepatica, commonly known as the liver fluke, is a zoonotic trematode that causes significant production losses to the ruminant livestock industry globally (Beesley et al., 2018). The annual economic impact of fasciolosis on the Australian sheep meat industry alone has risen from $25 million in 2015 to $38.3 million in 2022, ranking it as the 13th most important endemic disease of sheep according to recent Meat and Livestock Australia (MLA) estimates (Animal Health Australia, 2024; Shephard et al., 2022). Production losses are caused by extensive liver damage and haemorrhaging caused by migrating immature flukes, which impacts animal growth rate, body condition and

reproduction (Lalor et al., 2021; Shephard et al., 2022). In Australia, liver fluke is predominately confined to the South-eastern regions of New South Wales (NSW) and Victoria that experience high rainfall (>600 mm annually), and some irrigated areas (Brookes et al., 2024; Vyas et al., 2025). Approximately 51.3% (40.6 million) of Australia's national sheep herd and 34.5% (10.5 million) of cattle are raised in these liver fluke endemic regions (Meat and Livestock Australia (MLA), 2024a, 2024b).

Globally, livestock producers are highly dependent on chemical treatments to manage liver fluke infections, particularly triclabendazole (TCBZ) due to its unique high efficacy against both adult and early immature flukes within one week post infection (WPI) (Beesley et al., 2018; Boray et al., 1983; Kelley et al., 2016; Lamb et al., 2022). There are increasing reports of natural field resistance to TCBZ, having been found in over 11 countries since the first discovery in Australia in 1995 (Kelley et al., 2016; Overend & Bowen, 1995; Uthayakumar et al., 2025). Alarmingly, TCBZ remains the only chemical registered against human infection, which is a significant concern in areas where zoonotic infection is hyperendemic (Gandhi et al., 2019). These factors combined underscore an urgent need for livestock producers to adopt sustainable integrated parasite management (IPM) programs that increase the use of non-chemical control strategies to supplement and extend the lifetime of existing drugs. These programs rely on producers understanding the seasonality of infection, having the capacity and willingness to identify and fence off high risk snail habitats, and exploiting weaknesses in the parasite life cycle, such as spelling wet areas during summer to reduce pasture contamination with infectious cysts, to enhance chemical control (Boray & Love, 2017).

Liver fluke has a complex, indirect life cycle that requires both a definitive mammalian host and intermediate freshwater lymnaeid snail (Gastropoda: Lymnaeidae) host to complete its development, which presents both opportunities and challenges for IPM programs (Howell & Williams, 2020; Vázquez et al., 2018). The life cycle and epidemiology of fasciolosis is intricately linked to environmental factors, particularly the availability of fresh water and sustained temperatures above 10°C for the emergence and reproduction of susceptible lymnaeid snail species, and for larval development within the snail (Boray, 1969; Clunies Ross & McKay, 1929). In Australia, *F. hepatica* distribution has historically been limited to the presence of susceptible snails from the native *Austropeplea* genus (Boray, 1964). Invasive snails, namely *Pseudosuccinea columella* (formerly *Lymnaea columella*) and *Orientogalba viridis* (formerly *Lymnaea viridis* and *Radix viridis*), were first detected in urban areas of NSW in the early 1970s, and have since been found in livestock grazing regions in NSW, Queensland and Victoria (Boray, 1978; Boray et al., 1985; Chalmer & Kendrick, 1975; Molloy & Anderson, 2006; Ponder, 1975; Ponder et al., 2024; Salisbury et al., 1976). However, these studies only provide limited anecdotal updates on the distribution of these species since the initial incursion, with one laboratory study on *P. columella* collected from northern NSW undertaken to

understand its contribution to pasture contamination (Boray et al., 1985; Molloy & Anderson, 2006; Ponder et al., 2024). Continued epidemiological monitoring is essential to understand their specific environmental tolerances, distribution, and susceptibility to *F. hepatica* under Australian field conditions (Boray, 1978).

The current Australian recommendations for drench timing and choice are underpinned by studies conducted in NSW and Victoria during the 1960s and 70s (Boray, 1969; Boray et al., 1969; Boray & Love, 2017; Meek & Morris, 1979). Based on the findings of this foundational work, strategic drench timings were recommended to exploit weak points in the seasonality of the parasite. When average daily temperatures fall below 10°C, lymnaeid snails enter dormancy in the mud, reducing the likelihood of infection and subsequent contamination of pasture with infectious cysts (metacercariae) (Boray et al., 1969; Clunies Ross & McKay, 1929). Consequently, the level of metacercariae on pasture and hence infectious load, does not increase during the Austral winter (June – August). Australian producers remember and adhere to this strategic drenching schedule encapsulated by the phrase 'first frost, last frost'.

During a liver fluke drug resistance trial in the Southern Tablelands of NSW in June 2024 (Austral winter), numerous, active lymnaeid snails were found when average daily temperatures were regularly <7°C (Uthayakumar et al., 2025). The presence of intermediate snail hosts outside of the historical seasonal window challenges the foundations upon which these IPM strategies are based, including the 'first frost, last frost' treatment schedule. This finding therefore warrants urgent investigation to update our understanding of the current *F. hepatica* risk periods and the impact of susceptible invasive intermediate snail host species. The aim of the present study is to investigate the current abundance and diversity of the intermediate snail hosts of *F. hepatica* in the Southern Tablelands of NSW. The results from this longitudinal survey will assist in refining the current seasonal infective risk periods of liver fluke and inform evidence-based IPM strategies for producers in the Southern Tablelands of NSW that extend the lifetime of limited anthelmintics.

2. Materials and Methods

2.1 Farm enrolment and site selection

Farms endemic for *F. hepatica* were enrolled into the study after an information session was held locally in Gunning, NSW, in December 2024. Two sites at each farm were selected based on farmer suspicions, lymnaeid snail presence observed during the drug resistance trial in 2024 (Uthayakumar et al., 2025) and/or suspected to be favourable snail habitats based on their environmental characteristics (Boray, 1964).

2.2 Observational snail field sampling

To investigate the seasonality of the lymnaeid snails, sample collection visits were carried out every second month beginning in January 2025. The date and time of collection at each site were recorded at the start of the collection period. Photos of each study site were taken with smartphones and aerial shots with a drone to monitor habitat changes during each visit. At each study site, ten quadrats (32cm x 32cm) were randomly placed in ground with >10% water/wet mud to obtain a representative sample of snails at each site per visit (Perry, 2023). Snails within the surface mud of each quadrat were collected using featherlight forceps, paintbrushes and/or kitchen sieves, then placed into individual 5mL Eppendorf tubes labelled with the date, site, and quadrat number. Snails were immediately stored in RNAlater (1:10 tissue:fixative ratio) at 4°C until morphological assessment, after which, they were stored at -20°C until further processing.

2.3 Morphological snail identification

Snail species identification was first based on the presence or absence of morphological features, including the direction of the aperture (left or right) and presence of an operculum (Supp. Fig. 1; Boray & McMichael, 1961; Ponder et al., 2024). Individual snails were examined under an Olympus LG-PS2 stereo microscope, with left-handed snails separated from right-handed snails to exclude non-permissive intermediate hosts (Ponder et al., 2024). The respective abundance of left- and right-handed snails was recorded per quadrat. Further morphological assessment of the various shell characteristics was used to identify the right-handed lymnaeid snails as *Austropeplea* sp., *P. columella*, or *O. viridis* according to the Australian Freshwater Mollusc Interactive key and fact sheets (Supp. Fig. 1; Ponder et al., 2024). We elected to classify all snails from the genus *Austropeplea* as *Austropeplea* sp., to recognise unresolved taxonomy of the group (Puslednik et al., 2009).

2.4 Lymnaeid snail abundance and diversity

The average absolute abundance and standard deviation of lymnaeid snails per 0.1m^2 was calculated and recorded for each site per visit. The relative abundance of each permissive (right-handed) species was then calculated to permit comparisons of diversity over time. The Shannon diversity index was used to measure snail diversity for each visit to 3 decimal places using the following formula, grouping all left-handed snails as one taxa (Shannon, 1948):

$$H = -\sum[(p_i) * \ln(p_i)]$$

2.5 Detection of *F. hepatica* infection

To determine whether collected snails were infected with *F. hepatica*, total genomic DNA was isolated after removal of RNAlater via extensive washing with RO water. The tubes were gently inverted, with the RO water replaced at least 3 times. Within each quadrat, up to 5 snails were selected for processing. Snails that were visibly infected or morphologically identified as an invasive species were

preferentially included, and the remaining individuals were randomly selected to reach the per-quadrat maximum. Snails were inspected under an Olympus LG-PS2 stereo microscope for visible trematode larval stages (rediae and cercariae) using snail crushing and dissection methods described by Caron et al. (2008). Small snails <30mg were crushed whole. For larger snails, dissection of the digestive gland where trematode larvae typically reside was performed. The E.Z.N.A. Mollusc DNA kit (Omega Biotek, Australia) was used according to the manufacturer's instructions with the following modifications. The snail tissue was pulverised using a plastic micro pestle without liquid nitrogen and incubated for 1 hour at 60°C. Samples were then incubated at room temperature for 5 minutes with pre-heated (70°C) elution buffer. A second elution step was performed using the original column in a total volume of 50 µL elution buffer before the resultant DNA was stored at -20°C until further processing. A negative extraction control (ddH_2O) was included in each extraction run to detect contamination.

A set of genus-specific primers were used to specifically amplify *Fasciola* spp. at the ITS2 rDNA region. Individual qPCR assays were performed at a final volume of 20 µL, containing 10 µL of SSoAdvanced Universal SYBR Green Supermix (BioRad, Australia), and 2 µL of template DNA. Forward and reverse primers, SSCPFaF [S0754] (50-TTG GTA CTC AGT TGT CAG TGT G-30) and SSCPFaR [S0755] (50-AGC ATC AGA CAC ATG ACC AAG-30), were included at a final concentration of 100 nM. Samples were run in duplicate on a CFX96 Touch Real-Time PCR Detection System with the corresponding CFX Manager 3.1 software (BioRad, Australia). PCR reactions were initiated at 95°C for 3 min, followed by 40 cycles of 5 seconds at 95°C and 10 seconds at 60°C. All runs included a no template control (NTC; ddH_2O) to detect contamination and a positive control (DNA isolated from adult *F. hepatica*) to ensure assay specificity. Results were considered positive if both technical replicates matched the melt curve of the positive control and the mean C_T value was <35. All snail count and qPCR data were managed in Microsoft Excel (version 16.99.2) and analysed using GraphPad Prism (version 10.1.2, GraphPad Software, USA).

3. Results

3.1 Twelve favourable lymnaeid snail habitat sites were surveyed

A total of six farms from the Southern Tablelands of NSW were enrolled in the study, with two sampling sites selected per farm based on conversations with the producers (Figure 1). The latitudinal range of the sampled sites was 34°38'S - 35°07'S and the longitudinal range was 148°42'E - 149°19'E. The type of aquatic snail habitat at the selected sites included spring-fed wetlands (N = 10), a dam inflow (N = 1), and creek (N = 1).

3.2 Snail diversity and abundance changed over time

A total of 3,162 snails were collected across all 12 sites from January to July 2025, of which 1550 (49.01%) were identified as intermediate hosts for *F. hepatica* due to the right-handed opening and absence of an operculum. Following additional morphological identification training at the University of Melbourne in July 2025, the right-handed lymnaeids that had not yet been crushed or dissected for DNA isolation (92.77%) could be re-examined and identified to the species level. Out of the lymnaeids collected, the native *Austropeplea* sp. was most abundant (89.03%; N = 1380) and present at 10 of the 12 sites, followed by *P. columella* (1.74%; N = 27) found at A2, E2, and F2 (Figure 2 and 3). Only one *O. viridis* snail (0.06%) was found during the study, from site F2 during March (Figure 3). Of the total invasive species sampled, over half (59.3%; N = 16) were collected in July and were morphologically identified as *P. columella* (Figure 3). Site F2 recorded the highest abundance of lymnaeid snails per site during the study period, in July (N = 241; Figure 2). According to the Shannon diversity index, September had the highest species diversity (1.081), followed by July (0.803), May (0.640) and then March (0.609) (Table 1). Two sites (C1 and D1) remained dry up to the last sampling visit in September and therefore, no snails were found at either site. However, sampling remains ongoing (November 2025).

3.3 Detection of *F. hepatica* infection at 4 sites

Of the snails selected for DNA isolation (N = 338) and tested for *F. hepatica* infection to date (N = 214), 16 (7.48%) were confirmed as infected (Supp. Fig. 2). All were from the *Austropeplea* genus and were collected in March (Supp. Fig 2). Eight of the infected snails were from site B2 (50%), one from C2 (6.25%), four from D2 (25%), and three from E1 (18.75%) (Supp. Fig. 2). Analysis of these results is ongoing and outside the current scope of this manuscript but will be included in a final submission to *Veterinary Parasitology: Regional Studies and Reports*.

4. Discussion

This study investigated the current seasonality of the intermediate snail hosts of *F. hepatica* in the prime Merino sheep-producing regions of the Southern Tablelands of NSW through monitoring snail abundance and diversity, after discovering numerous, active lymnaeid snails outside the historical winter dormancy window during a drug resistant liver fluke trial in 2024 (Uthayakumar et al., 2025). With drug resistance confirmed in the region by Uthayakumar et al. (2025), knowledge of lymnaeid abundance changes over time is essential to refine IPM strategies. Nine of the twelve sites surveyed followed a seasonal abundance pattern consistent with historical epidemiological studies conducted by Boray et al. (1969) (Figure 2). In contrast, site F2 recorded the highest average abundance of lymnaeid snails (N = 24.1/0.1m^2, SD = 25.29) during the study thus far, in July when the mean weekly

air temperature was 7.95°C and the lowest temperature recorded was -3.1°C (Bureau of Meteorology, 2025b). Lymnaeid snails have recently been detected outside the critical 10°C threshold, in water temperatures as low as 2.8°C, at four sites across Argentina (Neira et al., 2025). Local and international reports of lymnaeid snails with the ability to withstand colder temperatures than what has been previously record suggest improved survival adaptations that may extend infectious risk periods for liver fluke in endemic regions. Further investigation of snails from these sites should be conducted to determine their environmental tolerances in relation to survival and cercariae shedding. These factors influence liver fluke seasonality and thus underpin updated IPM strategies.

From field observations, Site F2 is a typical spring-fed gully frequent found across regional grazing lands and appears no different to other spring-fed wetlands surveyed (Figure 4). Through utilising the knowledge of the lymnaeid abundance results from this study, producers of Farm F can preferentially grazing livestock in the F1 paddock over F2 over the winter months (June – August), to minimise the number of intermediate snail hosts of liver fluke potentially exposed to miracidia infection, or fence off this high risk gully area to break the life cycle, thereby reducing transmission risk to livestock and associated production losses. Due to time and resource constraints, it was not feasible to tour and survey all wet microhabitats for lymnaeids at each enrolled property. Instead, through opportunistic conversations, farm owners and staff were taught to identify favourable lymnaeid habitats and morphologically distinguish the right-handed intermediate hosts of liver fluke in the field. Farmers can apply these observational skills into their liver fluke IPM strategy farm-wide to monitor lymnaeid populations at high-risk sites throughout the year and formulate evidence-based management decisions, even after the conclusion of this study. These findings will be communicated to farmers in the region during an information day held at the local rural supplies store in Gunning in February 2026. By providing producers in the region with the outcomes and recommendations of the current study, evidence-based IPM strategies can be integrated to reduce reliance on chemical controls against *F. hepatica*.

At least one invasive lymnaeid snail was morphologically identified during the study at three sites (A2, E2 and F2) (Figure 3). *P. columella* was more abundant (1.74%) than *O. viridis* (0.06%), but both were significantly outweighed by the native *Austropeplea* sp. (89.03%) (Figure 3). This was also reflected in the low level of diversity indicated by the Shannon diversity index value approximately ≤ 1 for March, May, July and September (Table 1). The results from this study indicate that they are unlikely to be significantly increasing pasture contamination with infectious cysts, however, the increased abundance of *P. columella* during the cold July (59.3% of total invasive snails collected) is concerning. The study region experienced inconsistent and below average precipitation in the first half of this year and is evident through changes in green pasture availability observed in the aerial drone photos of site F2

across the year (Figure 4; Supp. Fig. 3). The invasive lymnaeid snail species are more tolerant of drier environmental conditions than *Austropeplea* which could have favoured the survival and proliferation of *P. columella* during July when the sampling sites were observed to be the driest. *P. columella* may be playing an important role in maintaining the life cycle of *F. hepatica* during long dry spells in the Southern Tablelands region, as has been previously found by Boray et al. (1985) at one site in the Northern Rivers region of NSW. This is the first study to monitor changes in *P. columella* and *O. viridis* populations across multiple seasons in Australia. It provides valuable baseline data for future surveillance studies conducted over multiple successive years (≥3 years) confirm the impact of *P. columella* on local infection dynamics.

There are two dynamics that need to be considered for this finding of lymnaeid snails outside the historical risk window to impact liver fluke seasonality: (1) If snails are present, at what time of year and at what temperature; (2) if the *F. hepatica* larvae can develop and release metacercariae. Detection of *F. hepatica* larvae using qPCR in this study has confirmed infected snails from sites B2 (50%), C2 (6.25%), D2 (25%), and E1 (18.75%) thus far. They were all collected in March and from the *Austropeplea* genus which is unsurprising due to their greater relative abundance. A laboratory study investigating the larval shedding from snails collected from positive sites under different environmental conditions (temperature, moisture, and pH) is required to determine the current thresholds required to confirm changes to the *F. hepatica* transmission risk window.

From combining the abundance, diversity and positive *F. hepatica* larvae detection results from this study, the following IPM recommendations to break the life cycle can be made. The high-risk areas for *F. hepatica* transmission should be fenced off, and spelled over summer because exposure to temperatures above 35°C for 2 weeks will kill infectious cysts (Boray, 1969; Clunies Ross & McKay, 1929). Adult cattle (>2years) that have developed age-related immunity to liver fluke can be rotated onto higher-risk pastures, over more vulnerable animals (calves and sheep). These evidence-based, non-chemical management strategies can help producers reduce their reliance on anthelmintics and help to preserve the remaining efficacy (Boray, 1969; Clunies Ross & McKay, 1929).

Limitations of this study include the two-month period between sample collections visits due to time, economic, and resource constraints. This means that changes in lymnaeid population dynamics in response to unusual climatic conditions between visits are unable to be captured, and thus only a general trend can be reported. Increasing the frequency of sampling trips to at least once a month would improve the precision and enable more detailed seasonal mapping of changes in lymnaeid populations. Sample collection visits were conducted as close to the same date every second month for consistency. Future studies should also incorporate routine collection of water and soil samples at each site. Analysis of these samples for basic physicochemical parameters (water hardness, water

temperature, pH, dissolved oxygen) together with qPCR screening for *F. hepatica* eDNA would provide complementary data on microhabitat suitability and enable detection of free-living or environmental liver fluke stages (Rathinasamy et al., 2021).

Morphological identification of snails is hindered by their phenotypic plasticity (Pfenninger et al., 2006). DNA samples extracted during the present study will be used for downstream molecular identification using Oxford Nanopore sequencing technology at the University of Melbourne to resolve the *Austropeplea* genus (Chen et al., 2025). Mapping the genetic sequence of the various lineages of *Austropeplea* in Australia will assist future monitoring and surveillance of lymnaeid distribution to be more efficient and accurate, in turn make staying up to date with current *F. hepatica* risk periods as climatic conditions change long term easier and therefore IPM strategies.

In conclusion, this study provided the first longitudinal data on lymnaeid snail abundance and diversity, in a prime Merino producing region, the Southern Tablelands of NSW. Alongside changes in the abundance of the native *Austropeplea* sp. throughout the year, we recorded the first seasonal study on the invasive *P. columella* and *O. viridis* outside of urban areas. This study revealed a site which seasonal abundance pattern of intermediate snail hosts deviated significantly from historical trends and therefore warrants further investigation into the area and intermediate snail hosts that are extending the seasonality because they may be contributing to the increased drug resistance. This study provides preliminary data to inform the current seasonal infective risk periods of liver fluke and evidence-based IPM strategies to preserve the remaining efficacy of anthelmintics as long as possible in the Southern Tablelands of NSW.

Acknowledgements

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5. Figure captions

Figure 1: Study area and sampling sites in the Southern Tablelands of New South Wales region (N = 12). Map shows approximate locations of two sampling sites at each of the 6 enrolled farms (A - F); scale bar indicates 20 kms. Water type of each favourable snail habitat sampled indicated in the legend. Aerial photos of sites were taken with a drone during May. Site locations approximated to maintain confidentiality. Figure created using Google My Maps, Google Earth and Canva.

Figure 2: Average absolute abundance of lymnaeid snails per 0.1m^2 at each sampling site (A1 – F2) in the Southern Tablelands of NSW, across five bi-monthly field visits in 2025. Bars are colour coded by month as shown in the legend; error bars represent the standard deviation among 10 quadrat replicates sampled at each site, each visit. Figure created in GraphPad Prism.

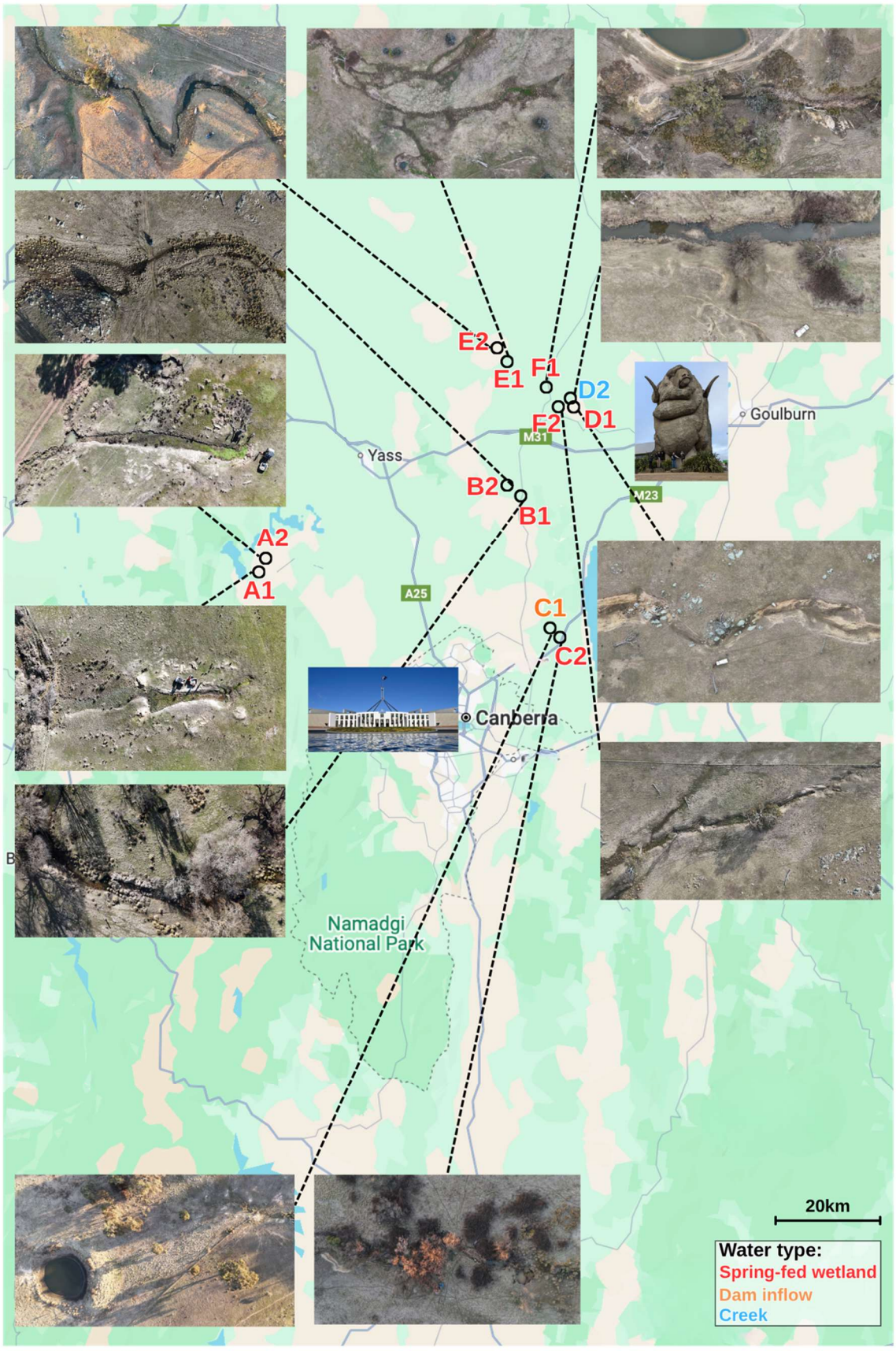
Figure 3: Comparison of snail population diversity at each sampling site (A1 – F2) in the Southern Tablelands of New South Wales, across five visits in 2025. Stacked bars show the composition percentage of each snail taxa collected at each site during each visit (January, March, May, July, September). Colours indicate taxonomic group as shown in the figure legend. Grey = left-handed snails (recorded but are not permissive intermediate hosts of liver fluke and were therefore not speciated further for this manuscript; pink = *Austropeplea* sp; green = *Pseudosuccinea columella*; blue = *Orientogalba viridis*. Brackets in the legend indicate permissive hosts of liver fluke and whether each is a native or invasive species. Images of preserved shells in the figure legend from Ponder et al. (2024). Figure created in GraphPad Prism.

Figure 4: Comparison of lymnaeid intermediate hosts of liver fluke at each sampling site (A1 – F2) in the Southern Tablelands of New South Wales, across four field visits in 2025. Stacked bars show the composition percentage of each snail taxa collected at each site during each visit (March, May, July, September). The native intermediate snail hosts of liver fluke are from the *Austropeplea* genus (pink), while *Pseudosuccinea columella* (green) and *Orientogalba viridis* (blue) are invasive species. Images of preserved shells in the legend are from Ponder et al. (2024). Figure created in GraphPad Prism.

Figure 5: Seasonal changes in wet microhabitats favourable to intermediate hosts of liver fluke at sampling site F2, located in the Southern Tablelands of New South Wales, during 2025. Photos of sampling site F2, a spring-fed gully: (A) January; (B) March; (C) May; (D) July; (E) September. (A) ground view taken with a smartphone camera. (B – E) Aerial view captured with a drone; scale bar indicates 5 metres.

6. Figures

Figure 1



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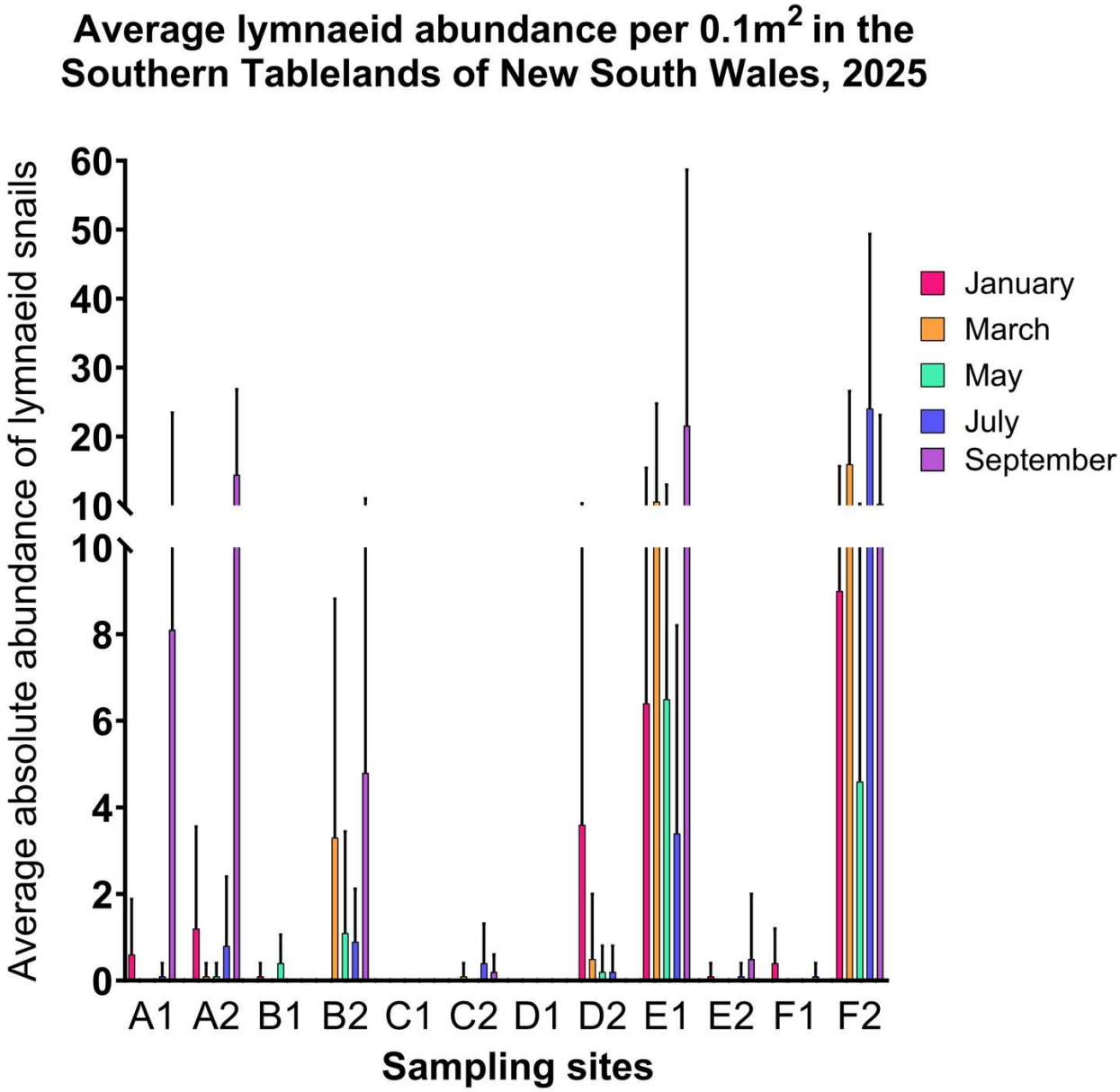

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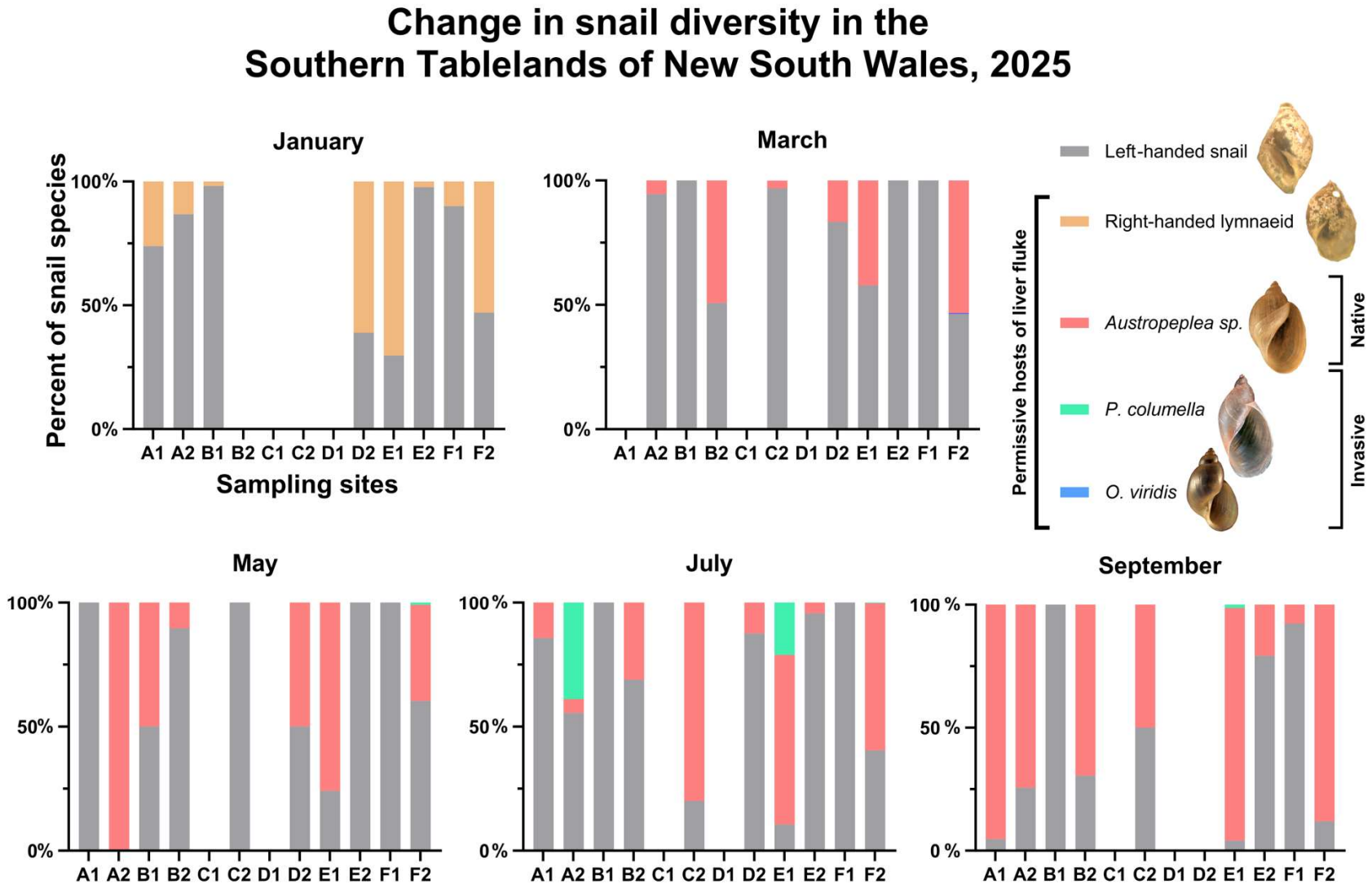

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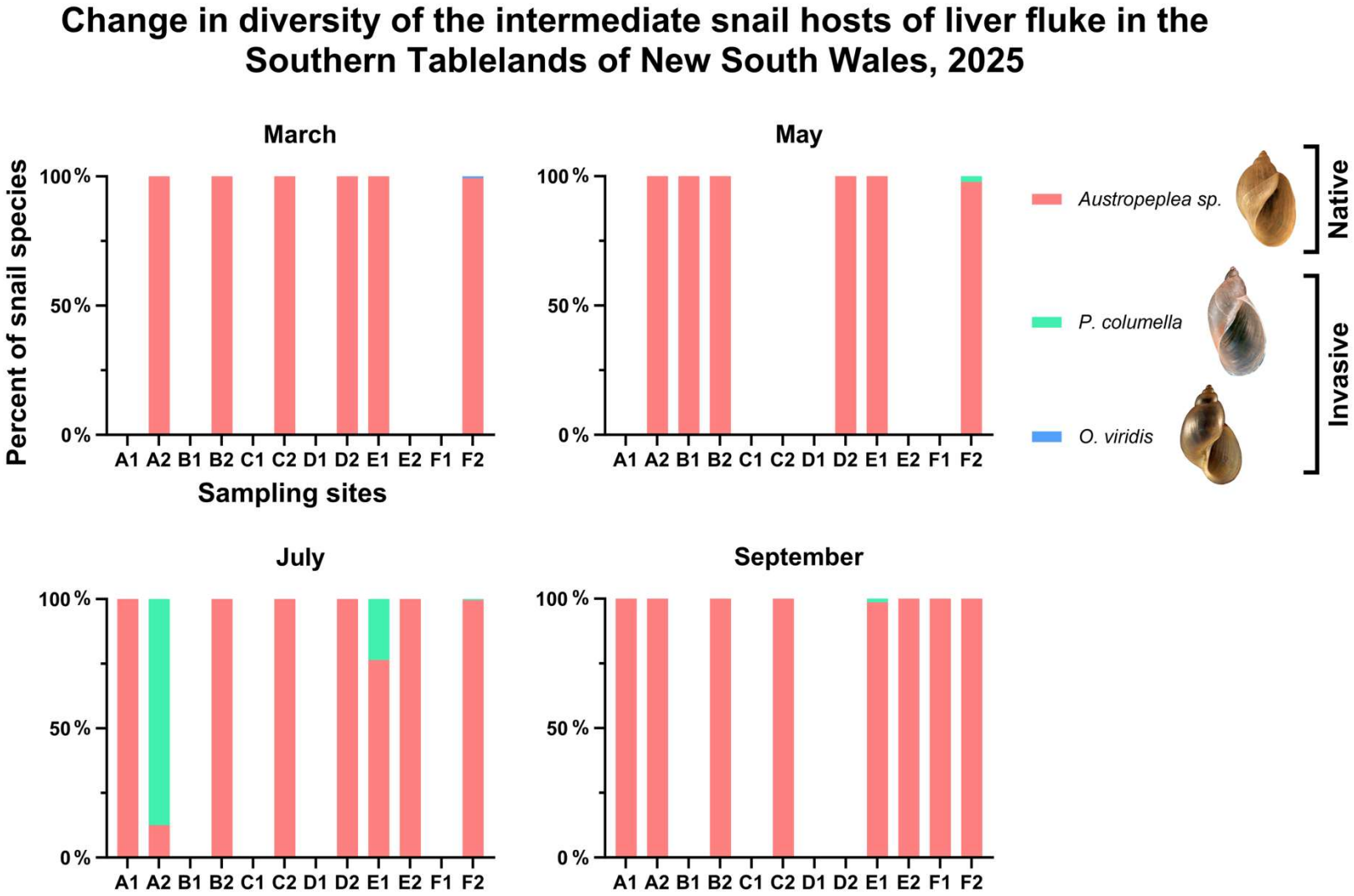

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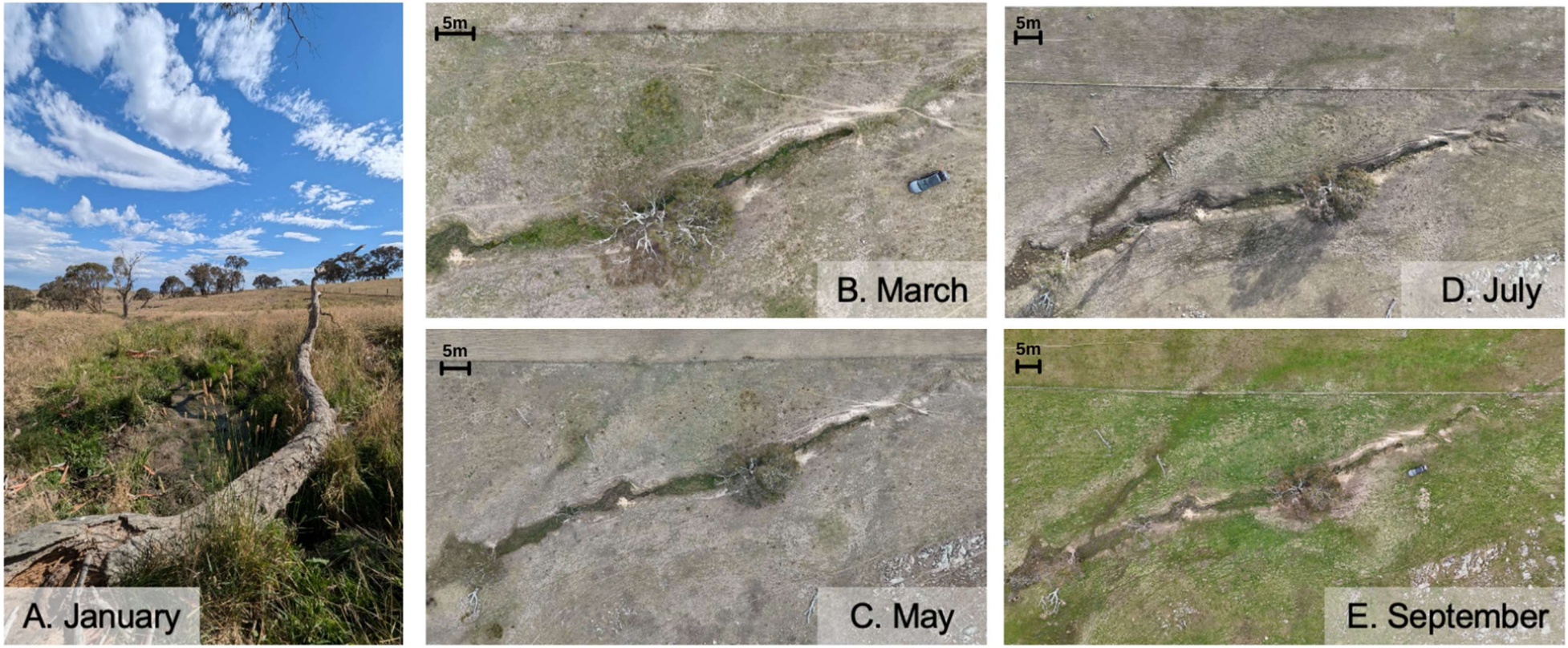

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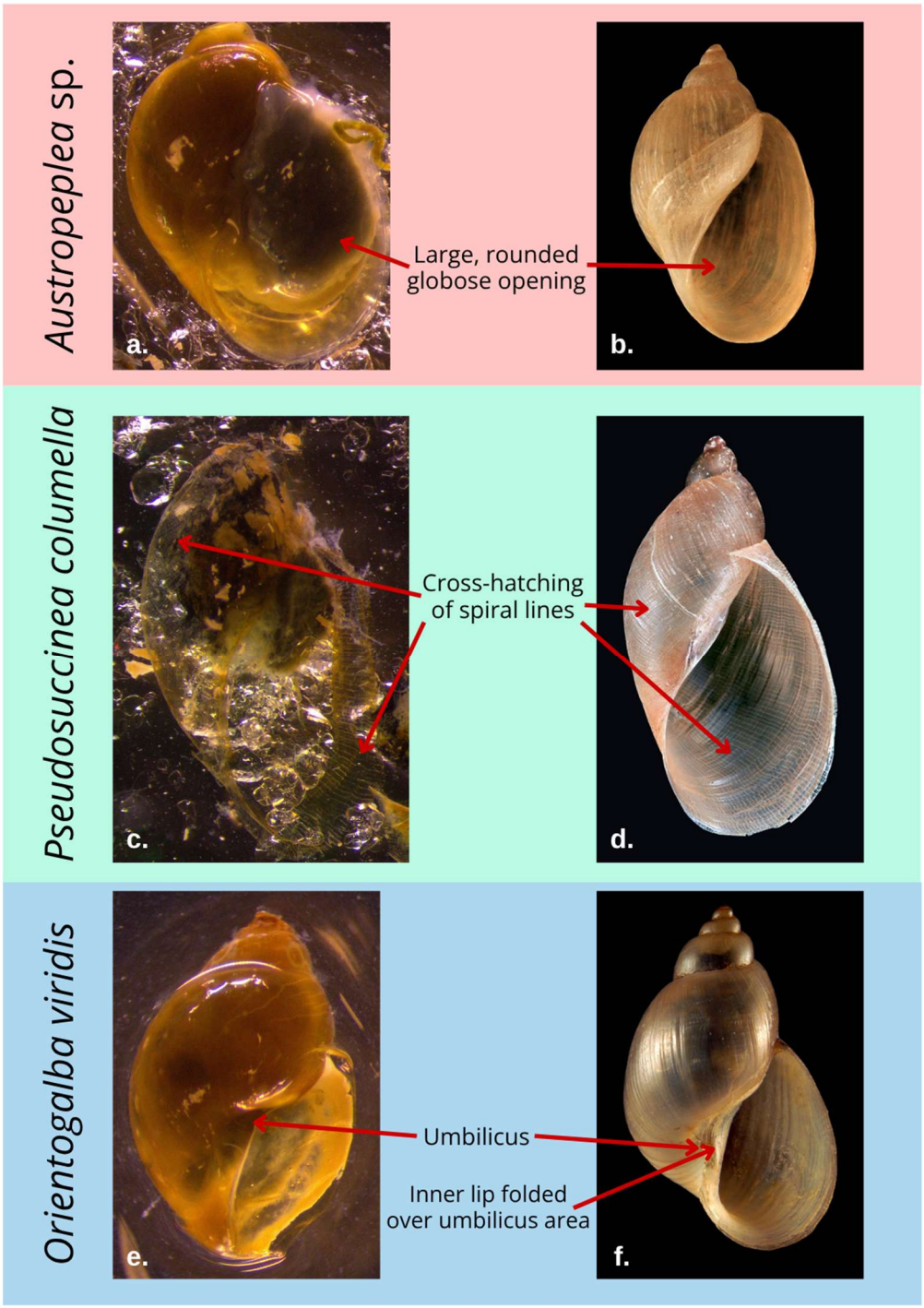
6. Tables

Table 1: Shannon diversity (H) of snail populations samples from 12 sites over four bi-monthly visits in the Southern Tablelands of New South Wales, 2025. Left-handed snails were grouped as one taxa because they are not permissive intermediate hosts of *Fasciola hepatica* and were therefore not speciated further.

Month	Shannon diversity index (H)
March	0.609
May	0.640
July	0.803
September	1.081

7. Supplementary figures

Supplementary Figure 1: Distinguishing morphological characteristics between the various intermediate snail hosts of liver fluke present in the Southern Tablelands of New South Wales. Snails on the left (a, c, e) contain the snail tissue and have been preserved in RNAlater then imaged under a Leica stereo microscope. Snails on the right (b, d, f) are images of preserved shells from Ponder et al. (2024). Figure created in Canva.



Supplementary Figure 2: *Fasciola hepatica* larvae detection by qPCR in lymnaeid snails from the Southern Tablelands of New South Wales. Results of individual snails presented as the species identification (RHL = Right-handed lymnaeid; A = *Austropeplea* sp. P = *Pseudosuccinea columella*; O = *Orientogalba viridis*), site collected from (A1 – F2), mean C_t value, and qPCR result ('N is negative with *F. hepatica*; 'Y' is positive with *F. hepatica*). Results were considered positive if both technical replicates matched the melt curve of the positive control (DNA isolated from adult *F. hepatica*), and the mean C_T value was <35. Presented by sampling month: (A) January; (B) March; (C) May; (D) July.

A)

January

Snail ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Morphological ID	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL
Site	A1	A1	A1	A1	A1	A1	A2	A2	A2	A2	A2	A2	A2	A2	A2	B1	D2	D2	D2	D2	D2	D2	D2	D2	D2	D2	D2	D2	D2	D2
Mean Ct	39.02	36.93	36.68	37.32	37.10	36.12	30.58	30.63	35.08	31.70	35.44	34.73	33.47	34.60	31.02	37.42	37.24	37.40	33.89	35.17	34.86	36.42	37.40	34.26	33.77	34.08	35.30	34.53	39.61	36.38
qPCR Result	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Snail ID	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Morphological ID	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL
Site	D2	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E2	F1	F1	F1	F1
Mean Ct	37.51	36.33	37.14	37.46	35.69	37.52	36.07	35.87	37.93	35.36	37.79	36.81	35.83	38.12	35.36	39.74	0.00	38.70	0.00	4.74	37.40	38.34	37.84	0.00	0.00	39.08	36.21	37.50	36.17	37.55
qPCR Result	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Snail ID	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
Morphological ID	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL	RHL
Site	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2
Mean Ct	36.00	34.64	36.36	36.02	38.73	36.59	36.51	36.63	36.54	0.00	37.33	0.00	0.00	1.33	37.07	0.00	35.53	37.83	37.11	38.98	37.21	0.00	0.00	38.02	38.72	37.64	38.34	0.00	38.63	37.09
qPCR Result	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Snail ID	91	92	93	94	95	96	97	98	99	100																				
Morphological ID	A	A	A	A	A	P	A	A	A	A																				
Site	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2																				
Mean Ct	39.35	35.92	34.74	35.85	33.99	39.72	0.00	38.94	38.16	36.24																				
qPCR Result	N	N	N	N	N	N	N	N	N	N																				

Key: RHL Right-handed lymnaeid; A *Austropeplea* sp.; P Pseudosuccinea columella; O Orientogalba viridis; N Negative (N); Y Positive (Y)

B)

March

Snail ID	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130
Morphological ID	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Site	A2	B2	B2	B2	B2	B2	B2	B2	B2	B2	B2	B2	B2	B2	B2	B2	B2	B2	B2	B2	C2	D2	D2	D2	D2	D2	E1	E1	E1	E1
Mean Ct	39.08	35.05	39.22	35.90	36.71	37.62	36.51	38.49	37.49	38.09	36.45	36.38	10.77	10.37	24.18	27.17	24.29	9.88	10.09	29.31	32.55	29.62	34.72	34.03	31.00	33.71	33.12	37.46	27.12	37.02
qPCR Result	N	N	N	N	N	N	N	N	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	N	Y	N
Snail ID	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160
Morphological ID	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Site	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	F2	F2
Mean Ct	36.48	36.99	38.33	37.49	39.42	32.88	36.29	37.62	38.15	37.30	36.72	39.75	38.33	0.00	34.77	35.58	36.33	35.00	0.00	8.34	39.98	39.49	39.09	0.00	2.67	0.00	2.83	32.18	26.09	32.72
qPCR Result	N	N	N	N	N	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Snail ID	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190
Morphological ID	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	0	A	A	A	A	A	A	A	A	A	A	A
Site	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2
Mean Ct	29.92	33.78	36.90	35.46	1.92	35.87	27.49	33.61	12.17	25.47	35.76	23.20	26.50	36.14	34.88	35.85	35.59	37.58	37.99	34.52	37.66	35.80	35.64	36.32	36.74	33.05	36.70	35.61	33.96	33.85
qPCR Result	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Snail ID	191	192	193	194	195	196	197	198																						
Morphological ID	A	A	A	A	A	A	A	A																						
Site	F2	F2	F2	F2	F2	F2	F2	F2																						
Mean Ct	33.39	33.58	33.39	34.11	34.79	36.94	35.05	35.29																						
qPCR Result	N	N	N	N	N	N	N	N																						

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C)

May

Snail ID	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228
Morphological ID	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Site	A2	B1	B1	B1	B1	B2	B2	B2	B2	B2	B2	B2	B2	D2	D2	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1
Mean Ct	30.20	33.40	33.42	33.04	35.21	34.75	37.68	37.93	38.20	39.18	39.19	35.02	38.09	36.60	36.88	38.16														
qPCR Result	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N														
Snail ID	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258
Morphological ID	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Site	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2
Mean Ct																														
qPCR Result																														
Snail ID	259	260	261	262	263	264																								
Morphological ID	A	A	A	A	A	A																								
Site	F2	F2	F2	F2	F2	F2																								
Mean Ct																														
qPCR Result																														

D)

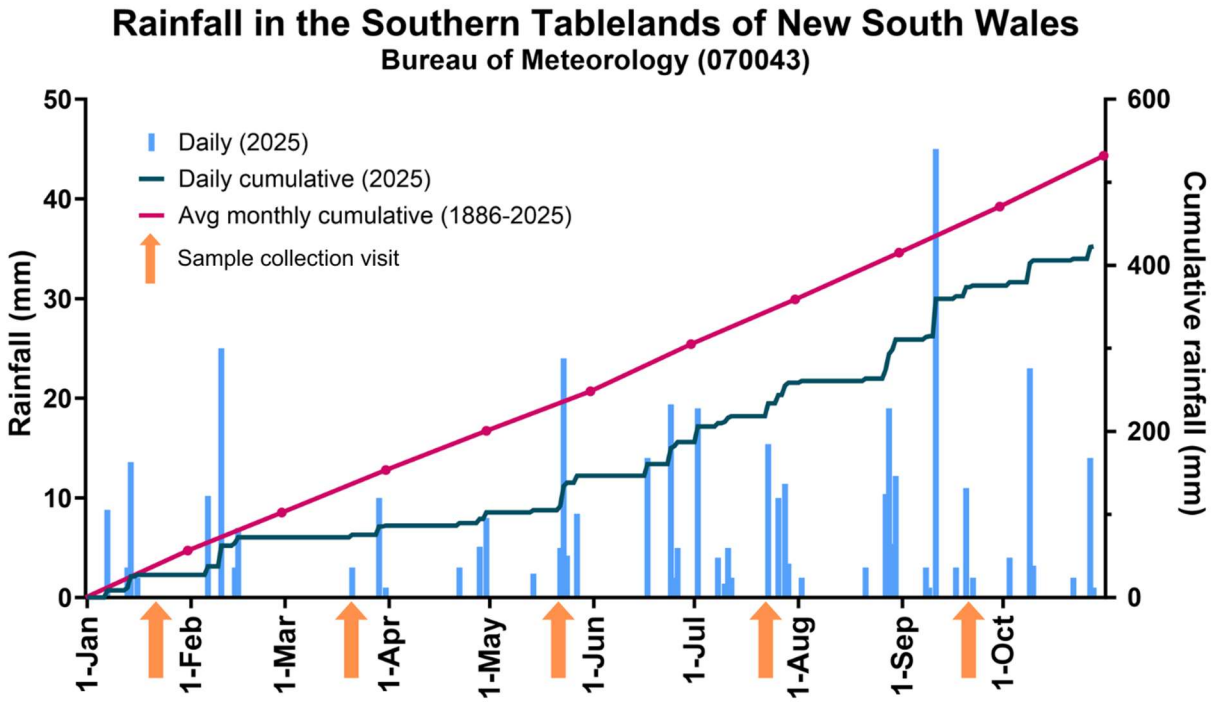
July

Snail ID	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294
Morphological ID	A	P	P	P	P	P	P	P	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	P	P	P	P	A	A	A
Site	A1	A2	A2	A2	A2	A2	A2	A2	A2	B2	B2	B2	B2	B2	B2	B2	B2	C2	C2	C2	C2	D2	D2	E1	E1	E1	E1	E1	E1	E1
Mean Ct																														
qPCR Result																														

Snail ID	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324
Morphological ID	P	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Site	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	E1	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2
Mean Ct																														
qPCR Result																														

Snail ID	325	326	327	328	329	330	331	332	333	334	335	336	337	338
Morphological ID	A	A	A	A	P	A	A	A	A	A	A	A	A	A
Site	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2	F2
Mean Ct														

Supplementary Figure 3: Annual rainfall recorded at Gunning Rural Supplies (Bureau of Meteorology station 070043), located in the Southern Tablelands of New South Wales. Blue bars = daily rainfall (mm); dark teal line = cumulative daily rainfall for 2025; dark pink line = average cumulative monthly rainfall (1886 – 2025) used for comparison. Orange arrows indicate dates of field sample collection visits. Created in GraphPad using data from Bureau of Meteorology (2025a) up to and included the 30th of October.



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