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Serpentoviruses in Free-Ranging Shingleback Skinks (*Tiliqua rugosa*) in Western Australia and South Australia, Australia

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ABSTRACT: Serpentoviruses are strongly associated with upper respiratory tract disease in captive and free-ranging bluetongued skinks (*Tiliqua* spp.). In Australia, bluetongue serpentoviruses were first reported in shingleback skinks (*Tiliqua rugosa*) with upper respiratory tract disease that presented to wildlife rehabilitation facilities in Perth, Western Australia. Since then, serpentoviruses have been detected commonly in captive bluetongued skinks from most areas of Australia, yet knowledge about the prevalence and distribution of these viruses in free-ranging bluetongued skinks, and other skink species, remains limited. Oral swabs were collected from 162 shingleback skinks from four areas in Western Australia and neighboring South Australia to screen for bluetongue serpentoviruses by PCR. The proportions of PCR positives were 0% (0/4) for Rottnest Island (a small island west of Perth, Western Australia), 3% (1/32) for the Shire of Kent (~5,600 km^2 in the southwest of Western Australia), 1% (1/91) from an approximately 250,000 km^2 area across South Australia and Western Australia, and 0% (0/35) from Mount Mary (~150 km^2 in the mid north of South Australia). Neither of the two PCR-positive shingleback skinks had overt signs of upper respiratory tract disease. These results are consistent with serpentoviruses occurring at a relatively low crude prevalence of 1.4% (95% confidence interval, 0.2–4.9%) across these areas, although the potential bias from sampling active and apparently healthy individuals may mean that this estimate is lower than the true prevalence. This contrasts with the high proportion of PCR positives reported in captive individuals. In the absence of experimental or observational data on viral clearance and recovery, *Tiliqua* spp. skinks that are intended for release into the wild should be housed with strict biosecurity to prevent interactions with captive individuals and screened to ensure that they are not PCR positive before release.

Key words: Disease, epidemiology, population sensitivity, wild.

INTRODUCTION

Free-ranging reptiles are susceptible to a number of key threatening processes, including habitat destruction, climate change, environmental pollution, unsustainable harvesting, introduced invasive species, and infectious diseases (Gibbons et al. 2000; Böhm et al. 2013). Studies on microbial infectious diseases in free-ranging reptiles have focused globally on mycoplasmas (Jacobson et al. 2014), onygenalean fungi (Allender et al. 2015; Allain and Duffus 2019; Allain et al. 2022), herpesviruses (Allain and Duffus 2019; Okoh et al. 2021; Allain et al. 2022), and ranaviruses (Allain and Duffus 2019; Adamovicz et al. 2020; Allain et al. 2022). Although there have been a number of studies on infectious diseases in Australian free-ranging reptiles, few have involved viral infections in nonaquatic species (O'Dea et al. 2016; Ariel et al. 2017; Hyndman et al. 2019; Maclaine et al. 2020).

The shingleback skink (or sleepy lizard or bobtail skink, *Tiliqua rugosa*) is a large (total length ca. 40 cm) omnivorous habitat generalist skink species (Norval and Gardner 2020), with a distribution across the southern half of Australia (Fig. 1). The species is classified as least concern by the International Union for Conservation of Nature Red List (Sanderson et al. 2017), although not all subspecies have this classification domestically (Heinrich et al. 2022), and arguably there are limited data to support the presumed viability of populations under threatening processes. Since the early 1990s, shingleback skinks in Perth, Western Australia (WA), have been affected by an upper respiratory tract infection associated

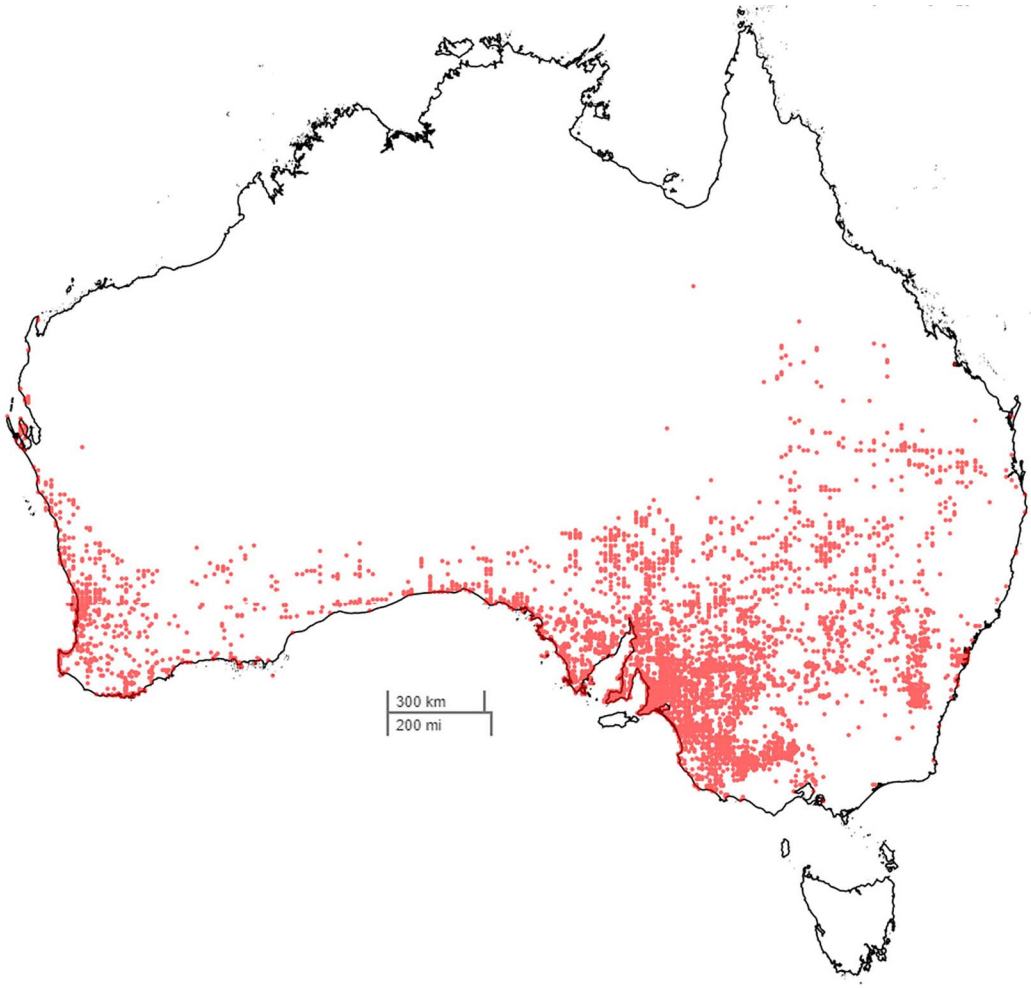

FIGURE 1. Map of Australia showing the reported distribution of free-ranging shingleback skinks (*Tiliqua rugosa*): Dots indicate observations of free-ranging shingleback skinks (Atlas of Living Australia 2023).

with a flulike disease referred to as "bobtail flu." In a 12-mo period during 2014–15, 95 cases were presented to a wildlife rehabilitation center in Perth for treatment of this disease (Kanyana Wildlife Centre 2015). Following early investigations into this disease (Lendon et al. 2011; Moller 2014), next-generation sequencing was used to discover and partially characterize a novel nidovirus that was significantly associated with clinical signs of respiratory disease in shingleback skinks (O'Dea et al. 2016). Of note, there were apparently healthy individuals that were PCR positive, suggesting that asymptomatic infection is possible. The name given to this virus was Shingleback nidovirus 1, which became the species name, but it was recently renamed to *Pregotovirus tiliquae* (subgenus: *Tilitovirus*; genus: *Pregotovirus*; subfamily: *Serpentovirinae*; International Committee on Taxonomy of Viruses 2023). Since then, serpentoviruses have been detected reasonably commonly in captive bluetongued skinks (*Tiliqua* spp.). They have not all been confirmed by phylogeny as belonging to the species *P. tiliquae* and so are hereafter referred to generally as "bluetongue serpentoviruses." Between September 2016 and September 2018, 61/170 (35.9%) of *Tiliqua* spp. samples tested at an Australian diagnostic laboratory were PCR positive for bluetongue serpentoviruses (Hyndman

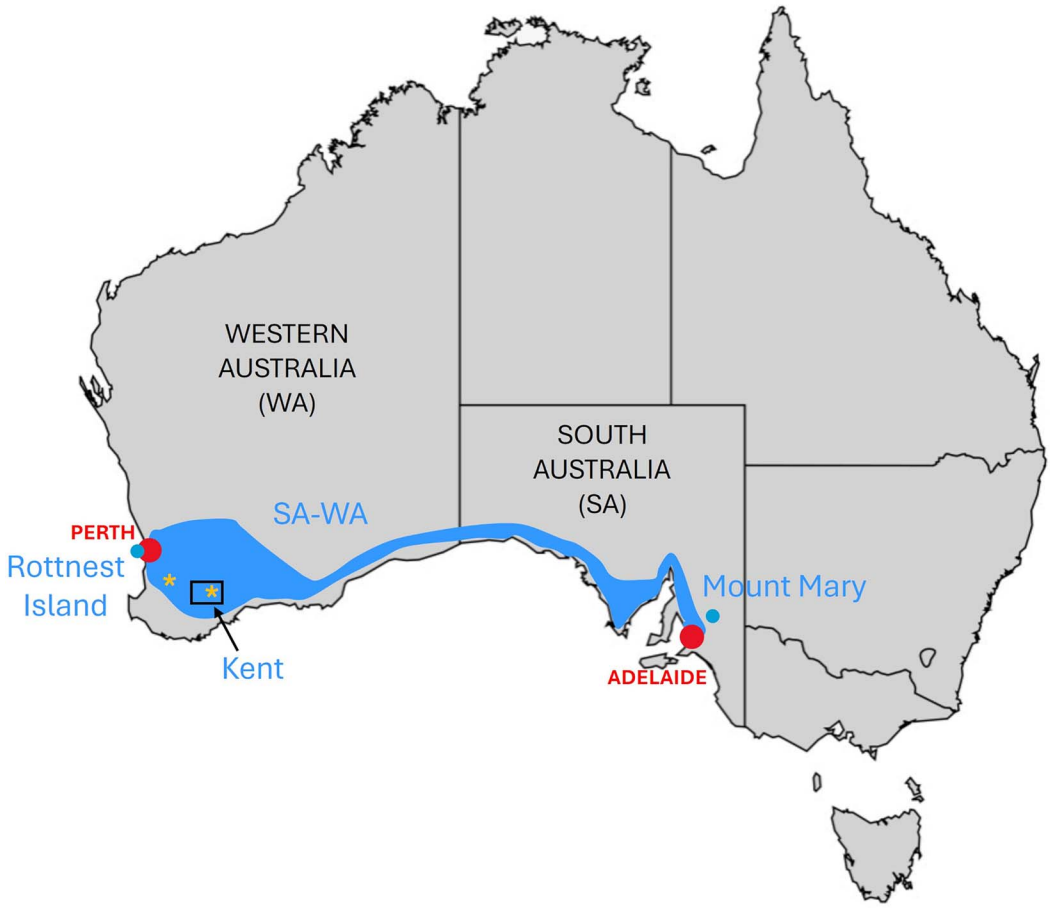

FIGURE 2. Map of Australia showing the four regions of skink sampling represented by shading, South Australia and Western Australia (SA-WA), Shire of Kent (Kent), or small dots, Rottnest Island in Western Australia and Mount Mary in South Australia. The large dots represent the major cities in South Australia and Western Australia. The asterisks represent the location of the two shingleback skinks that were PCR positive for the presence of bluetongue serpentoviruses; one from each of Kent and SA-WA.

and Shilton 2018). The majority of these samples were from captive animals, and many had signs of upper respiratory tract disease.

Shingleback skinks have close interactions with humans (as pets and in gardens) and are highly prized on the international pet market (Heinrich et al. 2022), but the epidemiology of nidoviral infections in this species remains unclear, particularly in free-living shinglebacks. Our study aimed to determine the presence of bluetongue serpentoviruses in free-living shingleback skinks from WA and South Australia (SA), Australia.

MATERIALS AND METHODS

Selection of sampling regions

Free-ranging shingleback skinks were sampled, using a cross-sectional study design, from four populations (Fig. 2 and Supplementary Material Figs. S1–S4): Rottnest Island (RI) in WA, the Shire of Kent (Kent) in WA, a large area across southern SA and WA (SA-WA), and Mount Mary in SA. Skink populations from RI were selected due to isolation from previously sampled mainland populations in the Perth region (O'Dea et al. 2016). Kent populations exist in fragmented habitat due to wide-scale vegetation clearing (Saunders 1989) and thus were also considered to be

relatively isolated from Perth populations. For the SA-WA site, a large expanse of the natural range of shingleback skinks in SA-WA was chosen, focusing on populations that would come into human contact along major highways. Mount Mary is a long-term research site for shingleback skinks (Norval and Gardner 2020), and there is anecdotal evidence of endemic skinks having upper respiratory tract disease.

Sample collection and assessment of health

Samples were collected from RI and Kent in the Australian summer (December–February) of 2018–19 and from the SA-WA and Mount Mary populations in the Australian spring (September–November) of 2019. Animal searching occurred during daylight hours, focusing on the morning and late afternoon periods when shingleback skinks exhibit peak movement (Chapple 2003; Kerr and Bull 2006). Transects were surveyed by motor vehicle or bicycle or both; search areas surveyed on foot used a meandering transect technique. Shingleback lizards observed during the survey were captured by hand in line with government-endorsed standard operating procedures (Department of Biodiversity, Conservation and Attractions 2017) and detained in situ for the duration of sampling and health assessment activities before immediate release. Sampling was conducted with approval from the Animal Ethics Committee of Murdoch University (permit no. RW3068/18) and the Animal Welfare Committee of Flinders University (E454/17).

Oral swabs were collected from each sampled animal, as described (O'Dea et al. 2016). An aseptic technique was used to collect saliva and mucus from the oropharyngeal area of individuals using sterile cotton-tipped applicators (Livingstone International, Mascot, New South Wales, Australia). Each swab tip was placed into 0.9% sterile saline solution in a 2-mL sterile screw-top tube.

A general health assessment was made of each individual to evaluate body condition and record any clinical signs suggestive of a disease state. Particular attention was paid to any signs of upper respiratory tract disease, such as pale membranes, labored breathing, and the presence of any oral mucus or ocular or nasal discharge.

Each lizard was photographed and marked so that it could be subsequently identified in case of recapture. Marking was done with a xylene-free paint marker in WA (Plummer and Ferner 2012) and via toe clipping in SA (Bull et al. 1981; Godfrey and Gardner 2017). Strict hygiene protocols were applied during handling and sampling of lizards, including the use of nitrile gloves and disinfection of all equipment and surfaces carried out between each sampling to prevent cross contamination of samples or viral transfer. For RI and Kent sampling, samples were immediately placed in a cooler with ice bricks until transferred to 4 C within approximately 8 h. Samples were then frozen at −80 C within 1 wk of collection until laboratory testing was undertaken. For SA-WA and Mount Mary sampling, samples were stored immediately at −18 C (SA-WA) or in liquid nitrogen (Mount Mary).

PCR testing

Extraction of RNA from samples was conducted using a viral RNA isolation kit (MagMAX 96, Thermo Fisher Scientific, Waltham, Massachusetts, USA), following the manufacturer's directions. For PCR, a one-step reverse transcription PCR kit (AgPath-ID, Applied Biosystems, Malaga, WA, Australia) was used. The master mix, primers (LnF: 5′-CGGAGTGGACAAGTCGTGAA and LnR: 5′-GGACTCAGTGCGGTGAGAAA), probe (Lnprobe: 5′-FAM-CGTCGCCGGTCAGACAGC GAGCC-BHQ1), and cycling conditions were as described by O'Dea et al. (2016). We used 2 µL of extracted RNA from each sample as a template in the PCR. A cycle threshold (Ct) value of <40, with a sigmoid curve, was defined as a positive sample. A Ct value of 40–45, with a sigmoid curve, was defined as an indeterminate result (O'Dea et al. 2016). A Ct value that was undetectable was considered negative. Bluetongue serpentovirus RNA was provided by one of the authors (T.H.H.) and was used as a positive control.

Epidemiologic analyses

Epidemiologic parameters were calculated using the RSurveillance package version 0.2.1, Sergeant 2020) in R (version 4.3.1, R Core Team 2023). Using the function *tp*, the true prevalence of bluetongue serpentoviruses was estimated for each population with a confirmed case of bluetongue serpentovirus (Kent and SA-WA), and the combined populations, using Blaker 95% confidence intervals (CIs). The sensitivity and specificity of the PCR test were assumed to be 0.9 and

1.0, respectively. Given the number of samples collected, the probability that a shingleback skink infected with serpentoviruses would be detected (the population sensitivity) was calculated using the function *sep.freecalc*. This was done for small (1,000) and large (100,000) populations, assuming low (1%) and high (10%) prevalences of infection.

RESULTS

The linear distances and sampling areas surveyed are listed in Table 1. The probability of detecting at least one PCR positive (the population sensitivity) was less than 50% for the RI sampling of shinglebacks, regardless of whether a low (1%) or high (10%) prevalence of infection was assumed. Assuming a low prevalence (1%) of infection, only the sampling of SA-WA reached a population sensitivity of >50%. When a high prevalence (10%) of infection was assumed, the population sensitivity was >95% (regardless of assumed population size) for the Kent, Mount Mary, and SA-WA sampling regions.

No skinks from RI, Kent, or SA-WA displayed signs of upper respiratory tract disease, including two PCR-positive skinks: one from SA-WA (Fig. 3A, B) and one from Kent (Fig. 3C). All shingleback skinks from the Mount Mary population were PCR negative for bluetongue serpentoviruses, including one animal that had bilateral conjunctivitis with mild serous ocular discharge (Fig. 3D). Given that the PCR-positive skink from the SA-WA population was in WA, there were no PCR-positive skinks from South Australia in this study.

Very low quantities of serpentovirus were detected in the two PCR-positive skinks. The Ct values for the PCR-positive skinks were 36.7 and 40.0 for the SA-WA and Kent animals, respectively.

DISCUSSION

Bluetongue serpentoviruses are commonly detected in captive Australian bluetongue skinks, yet our understanding of the distribution of these viruses in free-ranging populations is limited. In our study, bluetongue serpentoviruses were not detected in SA and they were detected in only two animals in this study: one from Kent and one from the WA portion of SA-WA. Note that samples collected from RI and Kent were held at 4 C for up to 1 wk, which may have resulted in false negatives. Further, the use of a highly specific probe-based PCR may have resulted in false negatives if the sampled skinks contained bluetongue serpentoviruses that were genetically divergent at the primer or probe annealing sites. Future studies should consider testing samples with broadly reactive PCRs (to detect divergent viral genotypes), plus specific quantitative PCRs to detect low-copy infections.

Neither of the two serpentovirus-positive animals had evidence of upper respiratory tract disease, and both were apparently healthy. Also, the high Ct values suggest that only small amounts of serpentovirus were being shed from each animal at the time of sampling. Taken together, these results are consistent with a mild, early, or clearing infection. In the study by O'Dea et al. (2016), 12% of infected individuals had no overt signs of disease; therefore, identifying apparently healthy serpentovirus-positive animals in the present study was not surprising.

Our crude prevalence across all four populations of 1.4% (95% CI, 0.2–4.9%) contrasts strongly with the 31% of shingleback skinks PCR positive among individuals presented to a Kanyana Wildlife Center in Perth (O'Dea et al. 2016) and the 35.9% PCR positive from a diagnostic service that screens captive bluetongued skinks (Hyndman and Shilton 2018). Note that these estimates were likely to have been affected by selection bias in all study populations. It is assumed that sick, rather than healthy, animals are disproportionately presented to wildlife rehabilitation centers and that sick animals presented to reptile veterinarians are tested for serpentovirus more often than are apparently healthy individuals. For free-ranging populations, the health status of animals may influence whether they are tested. Some sick individuals may seek roads

TABLE 1. Study populations, PCR results, and summary statistics of shingleback skinks (*Tiliqua rugosa*) from areas of South Australia and Western Australia (SA-WA), Australia, tested for bluetongue serpentoviruses. Skink populations were from Rottnest Island (a small island west of Perth, WA), the Shire of Kent in the southwest of WA, Mount Mary (approximately 150 km^2 in the Mid North of South Australia and an area approximately 250,000 km^2 area across southern SA-WA. Samples were collected from Rottnest Island and the Shire of Kent in the Australian summer of 2018–19 and from the SA-WA and Mount Mary populations in the Australian spring of 2019.

Study population	Survey area (km^2)	Linear kilometers surveyed	*n*	PCR positive	Estimated true prevalence (Blaker 95% confidence interval)	Population sensitivity[a] 1%/1,000	1%/100,000	10%/1,000	10%/100,000	Reference
Rottnest Island shingleback	0.063	426	4	0	NA[b]	3.56%	3.55%	31.5%	31.4%	This study
Shire of Kent shingleback	~5,600	2,513	32	1	3.5% (0.2–18.0%)	25.4%	25.1%	95.3%	95.1%	This study
Mount Mary shingleback	~150	590	35	0	NA	27.5%	27.1%	96.5%	96.3%	This study
SA-WA shingleback	~250,000	9,949	91[c]	1	1.2% (0.2–5.6%)	57.6%	56.1%	99.99%	99.98%	This study
All shingleback study populations			162	2	1.4% (0.2–4.9%)	NA	NA	NA	NA	This study
Kanyana shingleback[d]	NA	NA	83	25	Proportion of PCR positives: 30.1%	NA	NA	NA	NA	O'Dea (2016)
Captive bluetongued skinks[e]	NA	NA	170	61	Proportion of PCR positives: 35.9%	NA	NA	NA	NA	Hyndman and Shilton (2018)

[a] The probability of detecting at least one PCR positive (the population sensitivity) is presented, assuming low (1%) and high (10%) disease prevalence for both small (1,000) and large (100,000) shingleback populations.
[b] NA = not applicable.
[c] Twenty-five from SA and 66 from WA.
[d] Shingleback skinks presented to a wildlife rehabilitation center in Perth, Australia, in 2015.
[e] National diagnostic test results from bluetongued skink species (*Tiliqua* spp.) as of September 2018.

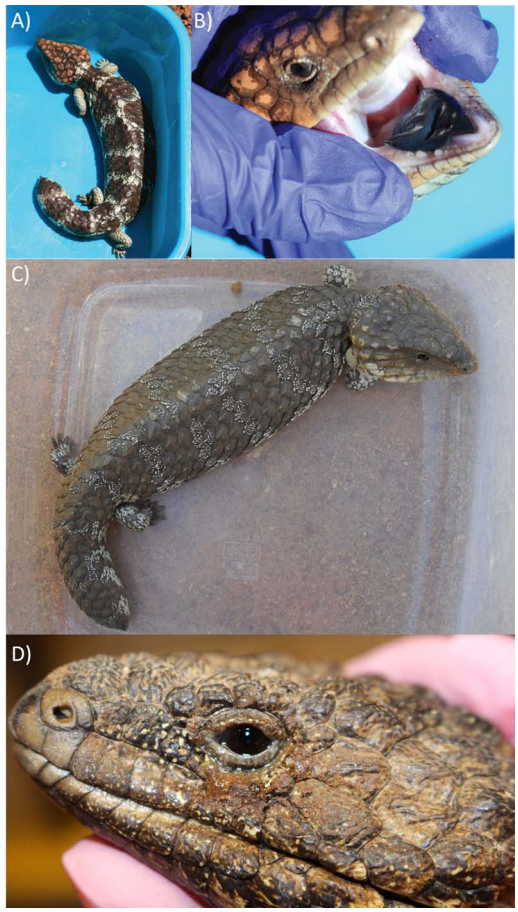

FIGURE 3. Shingleback skinks from South Australia and Western Australia (A and B) and the Shire of Kent, Western Australia, Australia (C) that were PCR positive for bluetongue serpentovirus. None had any apparent signs of upper respiratory tract disease. Shingleback skink from Mount Mary, South Australia, (D) with bilateral conjunctivitis and mild serous ocular discharge.

for basking, increasing the probability of being captured and tested, but other sick individuals may be less active, reducing the probability of capture and testing. Also, the sampling of animals during summer or spring rather than autumn or winter may have affected the estimated crude prevalence, as serpentovirus infections in free-ranging snakes from Southern Florida were found to be less common during the warmer seasons (Tillis et al. 2022). Further, the crude prevalence estimate involves aggregating populations across diverse landscapes and time periods, which is likely to affect the estimate in unpredictable ways, depending on host-pathogen-environment dynamics at the time of sampling. To derive a more nuanced understanding of the disease ecology of this important group of viruses, future studies should investigate the behavior of free-ranging shingleback skinks infected with bluetongue serpentoviruses.

In all four study regions, the climate may be hot and dry for consecutive months (Bureau of Meteorology 2023). Coupling this with serpentoviruses being enveloped (Parrish et al. 2021) means that environmental persistence of viable virus is likely to be brief. In an in vitro study on the thermal stability of ophidian serpentoviruses, virus held in culture medium at 35 C for a week typically had a reduction in infectivity by a factor of at least six million (Tillis et al. 2023). For these reasons, direct transmission of virus between skinks is the assumed mode of infection. In free-ranging shingleback skinks, different genotypes of *Salmonella enterica* were shown to transmit between animals according to the social network (Bull et al. 2012), and it is assumed that serpentovirus transmission would be similar. Another variable to consider in serpentovirus transmission is the persistence of viral shedding from infected animals. If persistent nidoviral shedding is common, then infections in free-ranging populations would be more likely to sustain themselves even in hot and dry conditions and where shingleback skinks exist in low population densities. The persistence of serpentovirus infections in shingleback skinks should, therefore, be explored as a priority.

Our study has shown that at the time of sampling, bluetongue serpentoviruses appeared to be uncommon in free-ranging shingleback skinks in SA-WA that are distant to Perth. Although it is tempting to conclude that serpentovirus is more common in captivity and peri-urban Perth populations than in the four study regions of our study, this conclusion would require unbiased surveys of the Australian captive and peri-urban Perth populations.

Also, other populations of free-ranging bluetongued skinks (not just shingleback skinks) should be tested to provide a broader overview of the prevalence of these serpentoviruses in Australia. Until then, it should be assumed that the Australian captive bluetongued skinks pose a biosecurity threat to their free-ranging cousins.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article is online at http://dx.doi.org/10.7589/JWD-D-23-00198.

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