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Influence of *Salmonella* specific bacteriophages (O1; S16) on the shedding of naturally occurring *Salmonella* and an orally applied *Salmonella* Eastbourne strain in bearded dragons (*Pogona vitticeps*)

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Abstract

This study determined the passage time and phage propagation time of salmonella specific phages, Felix O1 and S16, in 10 bearded dragons, based on re-isolation from cloacal swabs and faecal samples following oral administration, as a possible tool for reducing salmonella shedding. In Study 1, Felix O1 was administered orally for 12 consecutive days. Over 60 days, swabs were taken from the oral cavity and cloaca and qualitative *Salmonella* detection as well as salmonella quantification from faecal samples were performed. In Study 2, a phage cocktail (Felix O1 and S16) was administered to half of the tested animals. *Salmonella* (*S.*) Eastbourne was also given orally to all animals. Oral and cloacal swabs were tested as in Study 1, and faecal samples were collected for phage quantification. Various *Salmonella* serovars were detectable at the beginning of the study. The numbers of serovars detected declined over the course of the study. *S.* Kisarawe was most commonly detected. *Salmonella* titres ranged from 10^2 to 10^7 cfu/g faeces. The phages (Felix O1 and S16) were detectable for up to 20 days after the last administration. The initial phage titres ranged from 10^3 to 10^7 pfu/ml. The study shows that the phages were able to replicate in the intestine, and were shed for a prolonged period and therefore could contribute to a reduction of *Salmonella* shedding.

KEYWORDS
bearded dragon, PCR, phage, reptile, salmonella

1 | INTRODUCTION

Zoonotic transmission of *Salmonella* from reptiles to humans is a well-documented problem. Immunosuppressed individuals, especially children, are predominantly affected and develop clinical disease (Hatt et al., 2009; Murphy & Oshin, 2015; Woodward et al., 1997). Recent research indicates that these infections are often caused by insufficient hygiene practices following handling of pet reptiles. This can lead to direct or indirect environmental contamination, which can cause Reptile-Exotic-Pet-Associated-Salmonellosis

(REPAS; Pees et al., 2013). Numerous studies on *Salmonella* shedding have been conducted and demonstrated that reptiles carry and shed *Salmonella* intermittently (Lukac et al., 2015; Pasmans et al., 2008).

There are highly variable data on the prevalence of salmonella detection in reptiles reported in the literature, with prevalences ranging from 0.5% to 94% reported (Ebani, 2017; Mitchell & Shane, 2001). The great diversity in the detection rates is due in part to intermittent shedding and underlines the importance of the diagnostic methods used. Reptiles are often colonized with several *Salmonella* serovars simultaneously (Hydeskov et al., 2013), sometimes with up to four serovars per animal (Pees et al., 2013).

Although *Salmonella* are obligate pathogens in warm-blooded animals, it is hypothesized that some serovars are commensal bacteria in reptiles (Chiodini & Sundberg, 1981). One serovar with zoonotic potential is *S*. Eastbourne. This serovar has been detected in surveys in captive and wild reptiles all over the world. (Kikillus et al., 2011; Kuroki et al., 2013). *S*. Eastbourne has also been detected in multiples cases in Europe. Hydeskov et al. (2013) evaluated the zoonotic risk associated with reptiles and detected *S*. Eastbourne in 32% of the tested cloacal swabs. *S*. Eastbourne has been reported to cause infections in humans, especially in infants. Patients showed clinical signs of gastroenteritis and fever. *S*. Eastbourne was also isolated from a reptile in the same household as the *Salmonella* outbreak was detected (Hatt et al., 2009; Pees et al., 2013).

REPAS is a potential threat to human health, and numerous treatment trials have been conducted in the past in attempts to reduce or prevent the shedding of *Salmonella* in reptiles. However, none have led to long-term success so far. The Louisiana pet turtle industry bathed turtle eggs in gentamicin sulphate baths, with no significant reduction in *Salmonella* infection rates in the hatched turtles (D'Aoust et al., 1990). *Salmonella* serovars found in reptiles often display a high level of resistance against common antibiotics.

For example 81% of Salmonella isolates cultured from red eared sliders (*Trachemys scripta elegans*) showed a resistance to the antibiotic gentamicin (D'Aoust et al., 1990).

Other studies have found high levels of resistance against different types of antibiotics (Díaz et al., 2006; Silva-Hidalgo et al., 2014). Multi-drug resistance is also common among reptile *Salmonella* serovars. (Bertelloni et al., 2016).

Interest in phages and phage therapy has been increasing in recent years. The Eliava Institute in Georgia has been using phage therapy for half a century (Kutateladze, 2017). Several central European countries have now also started utilizing phages due to the rising problem of antibiotic resistance and the general aim to reduce the use of antibiotics (Górski et al., 2018).

When developing a new treatment method, it is also important to study the safety and efficacy of the new method. Loc-Carrillo and Abedon (2011) postulated a higher specificity of phages compared to antibiotics, due to their ability to lyse only bacteria with specific receptors. Other bacteria, especially the commensal flora, remain unaffected, while antibiotics potentially harm these bacteria as well. Fiorentin et al. (2005) suggested using a cocktail of different phages with different absorption receptors to reduce *Salmonella* more effectively.

Impacts

This study determined the passage time and phage preparation of *Salmonella* specific phages in bearded dragons. The study proved that phages can be detected for up to 20 days after the last administration. Intermittent shedding of salmonella could be detected as well as a reduction of *Salmonella* serovars in the animals. The amount of detected *Salmonella* in the faecal samples was highly variable. Nevertheless, the phages did have measurable effects on the *Salmonella*. The mean *Salmonella* shedding was reduced, and administration of a large quantity of phages led to a reduction in *Salmonella* shedding.

Several studies have been conducted with chickens. Most of these studies used phages as a food additive. They detected phages lead to an improvement of health status (Fiorentin et al., 2005) and there was a reduction of the horizontal transfer detectable (Lim et al., 2011). An additional positive effect of phages was a reduction of *Salmonella* colonization of the liver and gall bladder (Adhikari et al., 2017). The caecum also displayed a *Salmonella* titre reduction (Andreatti Filho et al., 2007), which explains why the environmental contamination through faeces with Salmonella was reduced (Fiorentin et al., 2005). However, this positive effect lasted only two days in one study (Andreatti Filho et al., 2007).

Bacteriophage Felix O1 displays a very high specificity and sensitivity in lysing *Salmonella* spp. (Kallings, 1967). In two studies 99.5% resp. 98.2% of the tested *Salmonella* strains were lysed by phage Felix O1 (Kallings, 1967; Welkos et al., 1974).

Marti et al. (2013) described a broad host range of the bacteriophage S16 against different *Salmonella* serovars without lysing other bacteria. This phage infects *Salmonella* using highly specific tail fibres that connect to the OmpC receptor on *Salmonella* surface. S16 also infects so called “rough” *Salmonella* strains that are defective in lipopolysaccharide (LPS) synthesis. This indicates that S16 does not need intact LPS to infect the bacteria. They tested 32 *Salmonella* strains and other bacteria. They discovered a greater susceptibility of *Salmonella* to S16, while other bacteria were resistant to this phage.

1.1 | Study aim

Phages have been described as a potential tool for *Salmonella* reduction and previous studies have demonstrated that they can be administered to bearded dragons and are then shed in the faeces (Renfert et al., 2019). This study therefore aimed to further investigate phage passage and shedding, as well as the impact of phage administration on the quality and quantity of *Salmonella* shedding in bearded dragons.

2 | MATERIALS AND METHODS

The study design included two parts. Study 1 was conducted to obtain information on the influence of bacteriophage Felix O1 on native *Salmonella* shedding in bearded dragons. Study 2 was then conducted to detect the influence of a phage cocktail (Felix O1, S16) on shedding of a *Salmonella* serovar administered with the phage cocktail. Parts of the results of Study 1 (phage administration, phage detection in swabs and faeces) have been described previously (Renfert et al., 2019). The animal studies were performed with the approval of the responsible authorities (Reg.-Nr.: TVV 15/17).

2.1 | Detection methods

For **qualitative reisolation of the phages**, swabs were placed in nutrient broth (Oxoid) containing a *Salmonella* reference strain (Anderson et al., 1977) and incubated at 37°C for six hours (Renfert et al., 2019). 10 μl of the broth were then plated onto a prepared "Blue Plate" (Rabsch, 2007) and evaluated as described.

For **quantitative phage assessment**, 1 gram of faeces was homogenized and serial diluted to 10^{-5} in buffered peptone water (Carl Roth GmbH + Co. KG). 10 μl of this dilution were plated onto BGA-Plates (Oxoid) which had been floated with the *Salmonella* reference strain. The plates were incubated at 37°C for 24 hr. Plaques were then counted and the phages were quantified (*Salmonella* and phages after adapting DIN ISO 18593:2009). Each sample was confirmed as phage Felix O1 by PCR (Renfert et al., 2019).

For **PCR confirmation of phage S16**, one primer pair was selected on the basis of GenBank Accession No. HQ331142 (Marti et al., 2013) created with Clone Manager 9 Professional Edition (Sci-Ed Software, 2016): S16-3′ Upper (CTGAGCCACGAATGAAGAC) and S16-3′ Lower (GACAAGGACGCTCTCAAC). This pair was combined with the ORF 2 primer pair for Felix O1. PCR protocols were used as described (Renfert et al., 2019).

For **qualitative *Salmonella* detection,** swabs were placed into a selenite cysteine broth and enriched at 37°C for 48 hr. A sample was then plated onto Rambach agar and OSCM plates and incubated at 37°C for another 24 hr. Single suspected colonies were serologically tested and classified according to the White-Kauffmann-Le Minor scheme.

For part 2 of the study, in order to identify and confirm *S.* Eastbourne, swabs were additionally plated onto Luria Bertani agar plates containing 90 μg/ml streptomycin (streptomycin-sulphate, Sigma-Aldrich, #S6501; inhouse preparation) in order to detect streptomycin resistant isolates. Colonies on this plate were transferred to a Rambach agar to detect streptomycin resistant *Salmonella* ssp. I, which were expected to be *S*. Eastbourne, and were then confirmed using the White-Kauffmann-Le Minor scheme.

In order to **quantify the amount of *Salmonella* shed in the faeces**, 100 mg of faeces were placed into 1 ml of buffered peptone water. After homogenization, the suspension was filtered into 9 ml of buffered peptone water and diluted to 10^{-6}. 100 μl of each dilution was plated onto XLT-4 plates (Oxoid) and incubated at 37°C for 24 hr. After incubation, the colonies were counted and quantified according DIN ISO 18593:2009.

For **phage resistance of the *Salmonella* serovars** the isolates were plated onto a nutrient agar (Oxoid) and 10 μl of the phage Felix O1 resp. the phage cocktail (containing Felix O1 and Phage Guard S®) were added. The plate was incubated at 37°C for 24 hr. Lack of lysis was seen in serovars resistant to the phage, while the presence of lytic plaques showed that the serovar was sensitive to the phage.

2.2 | Animals

Bearded dragons were obtained from commercial pet shops. Before the study began, each bearded dragon was examined clinically and a faecal parasitological examination was carried out. Only apparently healthy animals, in which no parasites were detected, were included. Swabs from the oral cavity and the cloaca as well as fresh faecal samples were collected and tested for the presence of *Salmonella* and *Salmonella*-specific phages using the methods described above.

The reptiles were housed individually in 2.0 × 0.8 × 0.6 m terraria for adults or 1.2 × 0.4 × 0.3 m for subadult animals. The ground substrate was children's playing sand, with stones and branches for environmental enrichment. The bearded dragons were fed six times a week with mixed greens (lamb's lettuce, chicory, meadow herbage, carrots) and twice a week with two to three medium house crickets. Food was supplemented with Korvimin ZVT® (WDT, Garbsen, Germany) daily and fresh water was always available. Neon lamps provided daylight 12 hr a day and a metal vapour lamp (Lucky Reptile Bright Sun UV Desert 50W, Waldkirch, Germany) provided additional lighting for 10 of those hours. The temperature in the terraria ranged from 25°C to 45°C under the metal vapour lamp.

Study 1 used eight male and two female (body mass ranging from 90 g to 341 g) bearded dragons. After a four week break, Study 2 was carried out with seven male and two female animals (identical to Study 1, body mass ranging from 104 g to 375 g). Four bearded dragons were randomly chosen and integrated into a non-phage group (animals 2–4 and 9). The other five animals were integrated into a phage group (animals 1 and 5–8).

Clinical monitoring included daily examination of the abdomen and oral cavity, and recording of abnormal behaviour, food consumption and faecal consistency. Appropriate hygiene measures were taken to prevent any cross contamination in the study.

Faecal samples were collected daily at 8 a.m., 12 a.m. and 4 p.m.

2.3 | Bacteriophages and *Salmonella*—propagation and administration

Phage Felix O1 was obtained from the RKI and used in Study 1. The phage was propagated on *S*. Paratyphi B strain B309 according to Rische (1973). Following propagation, the phage was filtered through 0.2 μm membrane filters (Sartorius Minisart, Merck,) and the titre was determined to be 1.2×10^9 pfu/ml as described (Rabsch, 2007). For Study 2, Phage Guard S® (Micreos Food Safety B.V.) as a commercially available phage cocktail consisting of bacteriophage Felix O1 and S16 (10^8 pfu/ml) was used.

Based on the evaluation of the pH stability of the Felix O1 phage (Renfert et al., 2019), a buffer preparation (Rennie Direkt Mikrogranulat® [Bayer]) was used to increase the gastric pH.2 ml of this buffer were administered orally, immediately followed by 2 ml of the Felix O1 suspension (Study 1) resp. 2 ml of Phage Guard S® (Study 2).

For Study 2, a *S*. Eastbourne-strain (antigen formula O9:He,h:H1,5) including a transferred plasmid for streptomycin resistance (pSL1344-3 of *S*. Typhimurium SL1344; GenBank No. HE654726.1), was obtained from the RKI (RKI-No. 18-545-pSL), incubated overnight at 37°C in glucose bouillon (Oxoid) and the bacteria were titrated in the bouillon to a concentration of 10^6 cfu/ml.

In Study 1, the phage suspension was administered daily for 12 consecutive days.

In Study 2, the phage suspension was administered to the animals in the phage group for three consecutive days. On the fourth day, both groups received 2 ml of the *S*. Eastbourne suspension p.o. From day 5 to 9 the phage cocktail and the *Salmonella* strain suspension were given alternately (Table 1).

2.4 | Sampling protocol

Before the start of each study, oral and cloacal swabs were taken for qualitative *Salmonella* analysis and a cloacal swab was tested to confirm that no *Salmonella*-specific phages were shed.

During the studies, all faecal samples collected were immediately prepared for quantitative *Salmonella* (Study 2: and phage) analysis. Swabs for phage detection were placed in a 1:1 mixture of glycerine (AppliChem GmbH) and glucose bouillon and frozen at −80°C.

For Study 1, samples were collected over a total of eight weeks, whereas for Study 2, samples were collected over a total of four weeks. For qualitative detection of *Salmonella* as well as qualitative phage detection, oral and cloacal swabs were collected three times a week during the period in which the animals were given a phage solution, followed by weekly sampling for the following period.

At the end of Study 2, three animals from each group were randomly chosen and euthanized. The bearded dragons were deeply sedated using ketamine (60 mg/kg i.m.; Ketamin 10%, Bremer Pharma GmbH) and medetomidine (0.25 mg/kg i.m.; Domitor, Vetoquinol GmbH) and then euthanized using T 61® (3 mg/kg i.c.; Intervet Deutschland GmbH). Necropsy was performed according to published standards (Mader, 2006).The gall bladder was carefully separated from the liver. The gall bladder, the liver and parts of the small and large intestine were placed into selenite cysteine broth for enrichment. The intestines were also incubated in glucose bouillons (prepared with *Salmonella* reference strain overnight at 37°C) at 37°C for 24 hr. 1 ml of the bouillon was then transferred to a sterile Eppendorf tube and centrifuged at 8,000 rpm for 15 min. 10 µl of the supernatant was then plated onto a BGA-plate and incubated at 37°C for 24 hr.

Details on treatments and sampling are provided in Table 1.

2.5 | Statistical analysis

Statistical analysis was performed using the program SPSS 22.0 (IBM). Based on the data evaluation, a standard distribution was not assumed. Therefore, the Mann-Whitney-U-test was used to determine significant differences between groups and over the course of the study. For the comparison of the *Salmonella* shedding titres, different time intervals were compared to each other, including 20-day-intervals (each third of the study), 30-day-intervals (first and second half of the study) as well as 14-day intervals, to overcome the varying shedding concentrations in the individual samples. Significance was assumed with $p \le .05$.

3 | RESULTS

3.1 | Phage detection

The detailed Felix O1 phage detection results from Study 1 have been published previously, to demonstrate the successful application protocol (Renfert et al., 2019). In summary, phages were first isolated from the cloaca three days after initiation of administration and remained detectable for a period of up to 24 days after the last administration. The phage titres ranged from 10^6 to 10^7 pfu/g faeces over the study period. The number of phages detected decreased during the course of the study until finally no more phages were detectable.

In Study 2, no phages could be found in the faeces in any animal before the start of phage application. Of a total of 27 faecal samples from the phage group animals, 12 samples were positive for phage Felix O1 and S16 and the phages were quantified. Of 25 cloacal swabs, 21 were positive. In these cases, the faecal samples from the same day were also positive and the number of phages in the samples could be quantified.

Phages were first isolated from two of the animals three days after they received the first phage cocktail, with phage titres increasing during the application period. The highest titres were found in the middle of the sampling period (day 8–day 15), then the titres decreased. The phages were detectable up to the last sampling day (day 20 after the last administration) in three of the five bearded dragons.

Intestine samples from all of the euthanized animals in the phage group were positive for both phages.

Details on the individual detection results are provided in Table 2.

TABLE 1 Application and sampling protocol for Studies 1 and 2

	Study day	-X	1	2	3	4	5	6	7	8	9	10	11	12	15	22	29	32	35	43	50	57	60
Study 1	Application O1 phage																						
	Oral/cloacal swab *Salmonella* detection																						
	Cloacal swab phage detection																						
	Faeces for *Salmonella* quantification																						
Study 2	Application phage susp. (phage group)																						
	Application *S.* Eastbourne (all)																						
	Oral/cloacal swab Salmonella detection																						
	Cloacal swab phage detection (phage group)																						
	Faeces for *Salmonella* (all) and phage quantification (phage group)																						
	Organs for Salmonella/phage detection																						

Note: In Study 1, 2 ml of the bacteriophage Felix O1 (titer 1.2×10^9 pfu/ml) were administered orally, following 2 ml of a buffer solution containing calcium and magnesium carbonate („Rennie Direkt Mikrogranulat®" [Bayer, Leverkusen, Germany] dissolved in 50 ml of drinking water).

In Study 2, 2 ml of the same buffer solution were administered before 2 ml of a *S.* Eastbourne solution (10^6 cfu/ml p. o.; given to all animals) or the administration of 2 ml of Phage Guard S® (consisting of bacteriophage Felix O1 and S16 [10^8 pfu/ml]; given only to the phage group).

TABLE 2 Phage detection in cloacal swabs and faecal samples, Study 2, phage group. Results of the qualitative (+/−) and quantitative (numbers) analyses of the samples. The displayed titre consists of the bacteriophages Felix O1 and S16 shed in pfu/g faeces

Treatment	Study day	Animal 1	Animal 5	Animal 6	Animal 7	Animal 8
Zero value		−/0E+01	−/0E+01	−/0E+01	−/0E+01	−/0E+01
Phage administration	3	+/0E+01		+/0E+01	9.09E+03	
	6			+/3.64E+04	−/0E+01	
	7			−/0E+01		
	8		+/2.27E+06	−/0E+01		
	9		+/4.27E+07	+/0E+01		+/0E+01
Post administration period	15	+/0E+01	+/1.18E+06	+/0E+01		+/0E+01
	16	+/4.82E+05				
	20			−/0E+01		
	22	+/0E+01		−/0E+01		+/5.64E+05
	23	+/7.27E+04		−/0E+01	−/0E+01	
	24			2.27E+05	−/0E+01	
	26				−/0E+01	+/4.91E+05
	27		−/0E+01	−/0E+01		
	28			−/0E+01	−/0E+01	
	29	+/4.45E+05		+/0E+01	+/9.09E+03	
	30				−/0E+01	
	31				−/0E+01	
Intestines		+	Not tested	Not tested	+	+

3.2 | *Salmonella* detection, Study 1

Initially, eleven different *Salmonella* serovars were isolated from the bearded dragons. Up to five different serovars were isolated from each individual reptile at the same time. The variability and number of the *Salmonella* serovars detected declined during the study. Initially on average 1.5 different serovars were found in each animal. This diversity dropped until the end of the application period to 0.9 serovars/animal, 0.7 in the post application period (from day 15) and finally only 0.6 serovars/animal on the last sampling day. This was partially caused by a reduction in the number of *Salmonella* positive samples: Initially 100% of the samples were positive, whereas during phage application and the post application period, *Salmonella* were only detected in 68% resp. 66% of the swabs.

S. Kisarawe O11:k:enx was detected at least once in seven of the ten animals, and was the serovar that was confirmed by far most often. Towards the end of the study, *S*. Kisarawe was detected with increasing frequency. This serovar was the predominant one in four animals. None of the 53 *S*. Kisarawe isolates were resistant to the phages used in this study.

One isolate from the oral cavity of animal 7 was confirmed to be *Salmonella*, although no O-antigens were expressed.

In two animals, the number of *Salmonella* serovars detected was reduced to a single one during the course of the study (*S*.O50:g,z51:-; *S*. O48:g,z51:-). These serovars were confirmed to be resistant to Felix O1. One bearded dragon remained completely negative for *Salmonella* after the initial determination of the serovars.

Although some *Salmonella* serovars were shed quite regularly, overall no serovar was isolated on all days. Some were isolated repeatedly over a certain time period.

Salmonella were more often detected in the cloaca than in the oral cavity, and those isolated from the oral cavity were often also confirmed in the cloacal swab samples, sometimes on different days.

Detailed information on the isolates are provided in Table 3.

Quantitative detection of *Salmonella* was positive in 77 of 121 faecal samples. The *Salmonella* titres measured were not constant during the course of the study, but ranged broadly from 10^2 to 10^7 cfu/g faeces. In one animal, no *Salmonella* were detected for the first 32 days followed by constant shedding. No faeces were obtained from one animal, despite a good appetite and apparent good health. Details on the individual sample results are shown in Table 4. Figure 1 plots *Salmonella* detection (median weekly shedding) and phage detection (in percent) during the study.

There were no changes in the mean *Salmonella* titres between days 14 and 20. However, when comparing *Salmonella* shedding during the first thirty days (first half of the study) with the last thirty days (second half) there was a reduction in the *Salmonella* average titres in the faecal samples ($p = .012$).

3.3 | *Salmonella* detection, Study 2

In general, the serovars that were detected at the end of Study 1 were again detected four weeks later in the initial samples of Study

TABLE 3 *Salmonella* serovars detected in Study 1. All animals were given phage Felix O1 orally on days 1–12. res, resistant to phage Felix O1. RDNC, react but do not conform with definite phage type. Sampling days: −1,1,3,5,8,10,12,15,22,29,36,43,50,57

Animal no.	Serovars isolated (isolation days)	
	Oral cavity	Cloaca
1	S. Guinea O44:z10:1,7 (−1)	S. Kisarawe11:k:enx (−1,1,3,5,8,15,22,29,36,50,57)
2		S. O58:lz13,z28:z6 (−1)
		S. O50:g,z51:- (res) (1,3,5,8,10,12, 15,22,29,36,43,57)
		S. Kisarawe11:k:enx (5)
3	S. O11:k:- (−1)	S. O11:k:- (−1)
		S. Offa O41:z38:- (−1)
4	S. O48:g,z51:- (res) (1,3,5,8,10,12,15, 57)	S. O48:g,z51:- (res) (1,3,5,8,10,12, 15,22,29,36,43,50,57)
	S. Guinea O44:z10:1,7 (3)	
5	S. Nima O28:y:1,5 (10,12,15)	S. Ouakam O9,46:z29:- (−1)
	S. Guinea O44:z10:1,7 (10)	S. Kisarawe O11:k:enx (1,43,50)
		S. Nima O28:y:1,5 (1,12,22,36)
		S. Guinea O44:z10:1,7 (3,15)
		S. O48:g,z51:- (res) (3,15)
6	S. Kisarawe O11:k:enx (8)	S. Adelaide O35:f,g:- (−1)
		S. Offa O41:z38:- (−1)
		S. Kisarawe O11:k:enx (1,3,5,8,15,22,29,36,43,50,57))
		S. Nima O28:y:1,5 (10)
7	S. Ouakam O9,46:z29:- (5)	S. Ouakam O9,46:z29:- (−1,29)
	S. sp. serologically rough (36)	S. Kisarawe O11:k:enx (1,3,5,8,10,12,36,43,50)
8	S. Ago 30:z38:- (3)	S. Ago 30:z38:- (−1)
		S. Kisarawe O11:k:enx (1,3,8,12,22,29,36,43,50,57)
		S. O11:k:- (8)
		S. O11:-:enx (12,15
		S. Apapa O45:m,t:- (10,12,15)
9		S. Typhimurium (−1,5)
		O 4,5:i:1,2 RDNC (−1,5)
10[a]	S. O48:g,z51:- (−1)	S. O48:g,z51:- (−1,10)
		S. Pomona O28:y:1,7 (−1)
		S. Kisarawe O11:k:enx (3,5,8,10)

[a]Died on day 17.

2. The consistent detection of *S.* Kisarawe was confirmed in the same animals.

S. Eastbourne was first isolated from a cloacal swab one day post administration.

Over the study period, *S.* Eastbourne was isolated from the cloaca in all animals from the non-phage group, and in two of five animals in the phage group. During necropsy, *S.* Eastbourne was also found in the intestines of one of the bearded dragons that was negative intravitam.

As in Study 1, *Salmonella* shedding was mostly intermittent, with the same serovars playing a dominant role. However, the variety of serovars initially found was lower (mean 0.9/animal).

One animal (animal 3) that became *Salmonella* negative during the course of Study 1 was positive for *Salmonella* during the initial screening for Study 2. Animal 2 stopped shedding *S.* O50 after receiving the phage cocktail, and only *S.* Eastbourne was detectable in further samples from this animal during Study 2. *S.* O48 was detected continuously in animal 4. Both serovars (*S.* O48 and *S.* O50) were resistant to Felix O1 and sensitive to S 16.The other isolated serovars were all sensitive to both phages. Detailed results are shown in Table 5.

Following necropsy, *Salmonella* serovars were isolated from the intestines of four of six bearded dragons. No *Salmonella* could be isolated from the gall bladders. In three cases, *Salmonella* serovars were isolated from liver tissue. In one case, the *S.* Eastbourne strain administered during the study was isolated. In both other cases, the serovar that was found in the liver was the same as the one predominantly isolated from the cloacal swabs. Isolate *S.* O48:g,z51:- was resistant to Felix O1 and sensitive to S 16. All other detected serovars were sensitive to Felix O1 and S 16 (details see Table 6).

Salmonella quantification was possible in 38 of 50 faecal samples (details see Table 7).

In three of the bearded dragons in the phage group, the *Salmonella* titre was reduced (10^4 to 10^5 cfu *Salmonella*/g faeces) compared to the mean titres shed in the same animals in Study 1. In two bearded dragons from the non-phage group, the amount of shedding varied considerably, and no fresh faecal sample was obtained from one animal during the study. The median shedding over four weeks was lower in the phage group ($6{,}8 \times 10^5$ cfu/g faeces) than in the non-phage group (1.6×10^6 cfu/g faeces). However, this effect was not statistically significant ($p > .05$).

3.4 | Clinical examination

During the study, the animals showed no changes in their behaviour or their food consumption. The clinical examinations revealed no changes in the health status of the bearded dragons. The subadult animals even increased their body mass.

During the course of Study 1 the subadult (smaller) bearded dragons demonstrated a slightly shorter defaecation interval as well as slightly more pasty/fluid faeces than the adults.

3.5 | Necropsy results

During Study 1, on day 17, bearded dragon 10 was found dead in its terrarium. Post-mortem examination including histology

TABLE 4 *Salmonella* quantification from faecal samples, bearded dragons 1–9

	Animal no.								
Trial day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
5						9.09E+02	2.91E+05	2.09E+06	
6						4.73E+04			
7						1.18E+06	1.18E+05	1.73E+06	
8						3.64E+05			
9							6.27E+05		
10	8.18E+03					6.91E+05		1.27E+05	
11						5.36E+05		1.73E+06	
12	1.00E+07							3.45E+08	
15							4.00E+06		
16						3.82E+07			
18					1.36E+06			4.45E+05	
21						9.09E+05			
22								6.18E+04	
23							6.45E+05		
24	1.73E+06					2.91E+04	3.73E+04		
26		2.00E+05					2.73E+06		
30						5.18E+04			
31	2.82E+06	3.27E+05			4.09E+06	7.64E+04	3.18E+05		
32	4.18E+06	2.27E+05					6.73E+07		
33	3.18E+06						4.82E+04		
35				1.55E+05		5.91E+05			1.55E+06
36							7.27E+05		
38	1.27E+04				2.45E+04		1.91E+04		
39	1.64E+04						1.53E+05	5.00E+04	
40	5.45E+05								
41						5.18E+04		6.91E+05	
43	3.73E+04			2.55E+05		1.55E+07	4.18E+06		
44		4.00E+04					1.00E+05		7.45E+06
45		1.00E+05		8.45E+05			1.27E+05		
50	2.00E+05	2.82E+04			1.00E+06		9.09E+04	2.73E+04	
51									
52						1.18E+05		3.18E+05	
53	6.36E+05	4.55E+05					2.73E+05		
58								1.36E+04	
59								4.64E+05	
60							1.18E+06	1.91E+05	8.18E+04

revealed an undetected infection with *Nanniziopsis guarroi* affecting multiple organs, which was not thought to be related to the study.

The scheduled necropsies at the end of Study 2 revealed sludge accumulation in the gall bladders in five of six animals. However, no gross changes were noted in the gall bladder walls of any of these animals, and no pathological findings were noted in the livers or intestines of any of the animals either.

4 | DISCUSSION

This study was conducted in order to obtain information on the influence of bacteriophages on the shedding of *Salmonella* serovars in reptiles. First results on the administration and shedding of phages in bearded dragons were used as a basis for the study. Phages were further detected in Study 2 to validate previously published data (Renfert et al., 2019).

4.1 | Phage detection

The Felix O1 phage isolation results from the first study (Renfert et al., 2019) already demonstrated that infective phages are able to pass through the stomach when gastric pH is increased by a buffer solution. The results found in Study 1 and Study 2, where a solution with two phages was used (see Table 2), are consistent with the initial results, and therefore demonstrate the reproducibility of the documented phage shedding behaviour. The last phage detection does not reflect the maximum shedding time, as this was just the last sampling day in the study. Since phages were isolated from the intestines of all of the animals that were necropsied, a longer shedding period is to be expected.

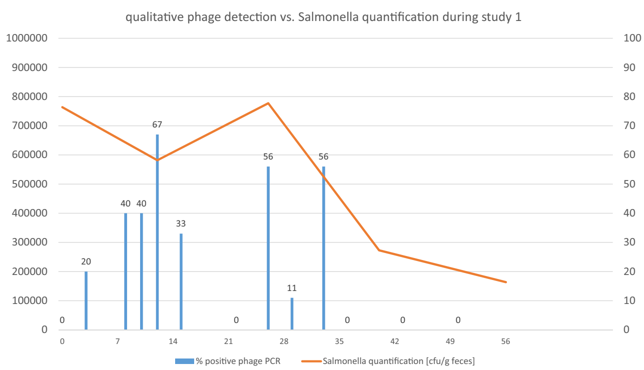

FIGURE 1 The median weekly Salmonella titers measured in fresh faeces compared to qualitative phage detection over a period of 56 days

The intermittent shedding of the phages, which was described in a prior study (Renfert et al., 2019), was also verified in this study.

Both phages used in this study—Felix O1 and S16—are lytic phages. They replicate exponentially, consistent with non-linear pharmacokinetics, in contrast with the fate of a drug, e.g. an antibiotic, administered to an animal (Hänggi, 2004). Various interactions with other bacteria or digestive fluids can influence the phage titre. Digestive enzymes or bile have been reported to cause phage reduction in an in vivo system (Joerger, 2003). Joerger (2003) also hypothesized that the viscosity in the intestine could influence the effectiveness of phages. The first barrier to intestinal colonization for orally ingested phages is the stomach with its low pH. To overcome this barrier, a buffer solution was used in both studies (Renfert et al., 2019).

Intermittent shedding has been previously described for *Salmonella* in reptiles (Lukac et al., 2015; Pasmans et al., 2008). It is possible that the same mechanisms that lead to the intermittent shedding of *Salmonella* also cause the intermittent shedding of specific bacteriophages or at least that the varying *Salmonella* concentrations in the intestine influence phage replication and shedding. This is supported by the fact that to some extent, the

TABLE 5 *Salmonella* serovars detected in Study 2. The animals in the phage group were given phage Felix O1 and S16 orally on days 1–3 and then alternating with S. Eastbourne up to day 9. All animals were given *S.* Eastbourne beginning on day 4. res, resistant to phage Felix O1. Sampling days: 1,5,9,15,22,29

		Serovars isolated (isolation days)	
	Animal No.	Oral cavity	Cloaca
Phage group	1		S. Kisarawe O11:k:enx (1)
			S. Eastbourne O9:e,h:1,5 (22)
			S. Nima O28:y:1,5 (29)
	5	S. Eastbourne O9:e,h:1,5 (5)	S. Nima O28:y:1,5 (1)
			S. Eastbourne O9:e,h:1,5 (5,22)
	6		S. Kisarawe O11:k:enx (1,9,15,22,29)
			S. Nima O28:y:1,5 (15)
			S. O48:g,z51:- (res) (15)
	7		S. Kisarawe O11:k:enx (1,5,9,15,29)
			S. O 48:g, z51:- (res) (5)
			S. Nima O28:y:1,5 (15)
	8	S. Kisarawe O11:k:enx (15)	S. Kisarawe O11:k:enx (1,5,9)
Non-phage group	2	S. Eastbourne O9:e,h:1,5 (9)	S. Eastbourne O9:e,h:1,5 (15,22,29)
	3		S. Nima O28:y:1,5 (1,5,15,22,29)
			S. Guinea O44:z10:1,7 (1,5)
			S. Eastbourne O9:e,h:1,5 (9,15)
	4		S. O 48:g, z51:- (res) (1,5,9,15,22,29)
			S. Eastbourne O9:e,h:1,5 (9)
	9		S. Kisarawe O11:k:enx (9)
			S. Eastbourne O9:e,h:1,5 (15,22,29)

TABLE 6 *Salmonella* serovars detected in liver, gall bladder and intestine after necropsy. res, only resistant to phage Felix O1

Group	No.	Liver	Gall bladder	Intestines
Phage group	1	S. Kisarawe O11:k:enx		
	7			S. Eastbourne O9:e,h:1,5 S. Kisarawe O11:k:enx S. Guinea O44:z10:1,7
	8			S. Eastbourne O9:e,h:1,5 S. Guinea O44:z10:1,7
Non-phage group	3	S. Eastbourne O9:e,h:1,5		S. Guinea O44:z10:1,7
	4	S. O 48:g, z51:- (res)		S. Guinea O44:z10:1,7
	9			

TABLE 7 *Salmonella* quantification during treatment with bacteriophages Felix O1 and S16

	Animal no.								
	Phage group					Non-phage group			
Study day	1	5	6	7	8	2	3	4	9
3								6.55E+06	
5					7.27E+03				
6							2.82E+04	4.55E+05	
7			1.27E+07						
8			9.91E+06			1.27E+06			
9				4.55E+07		4.18E+06			
13				3.00E+07					
14			1.18E+05		3.64E+04				
15			1.27E+06	4.55E+06		1.27E+07			
16	1.64E+07								
17			6.36E+06						
22		3.64E+05	1.91E+06		3.64E+05		5.45E+04		
23	2.27E+07	5.45E+04	1.82E+05		3.64E+05		1.36E+04	1.00E+05	
24			3.64E+04				6.73E+06		
25			6.36E+04						
26								1.27E+06	
27						2.27E+05			
28				2.82E+06			9.09E+05		
29	1.36E+07							2.36E+05	
30								1.45E+04	
32				2.27E+06					

phage detection rate varied in accordance with *Salmonella* shedding (Tables 2 and 4, Figure 1). The exact mechanisms behind this shedding behaviour have not yet been described. Possible effects of bacterial biofilms are discussed below. The varying results indicate that many individual factors influence the shedding of phages via the cloaca, as reported by Joerger (2003). The immune system is also a highly individual factor, especially in ectothermic reptiles, that might affect the phage titre in different ways. Since our study is based on data from ten (Study 1) respectively five (Study 2; animals out of the phage group) bearded dragons, these data should be considered preliminary and provide a "proof of principle". Additional studies with larger numbers of animals are necessary in order to fully understand the shedding of phages in bearded dragons, and to examine the influence of external and internal factors, such as temperature, feeding, age and husbandry conditions on growth and shedding of these viruses in reptiles.

4.2 | Qualitative *Salmonella* detection

The variability of *Salmonella* serovars was reduced by the end of Study 1. This reduction demonstrates the impact of the phage administration on the different serovars—the phage apparently eliminated the shedding of some, while others continued to be shed. In the present study, *S*. Kisarawe O11:k:enx seemed to be the predominant serovar. It was also the most common serovar during and after administration of the phage cocktail. Interestingly, *S*. Kisarawe was sensitive to Felix O1, as are all of the *S*. Kisarawe isolates that have been detected in the RKI (Rabsch, unpublished data). Despite this reported and tested sensitivity of *S*. Kisarawe to bacteriophage Felix O1 a (partial) resistance under in vivo conditions is still conceivable. It is also possible that the bacteriophages lysed *S*. Kisarawe, but an immediate recolonization from environmental contamination occurred, based on a selective advantage against other *Salmonella* serovars in the terraria.

Bacterial resistance against phages has been well-documented. Fiorentin et al. (2005) demonstrated that a continuous administration of bacteriophages could lead to bacterial resistance. A single point mutation of a phage adsorption protein on the surface of a *Salmonella* might create a selective advantage for this bacterial mutant (Gasiunas et al., 2014). Another way for bacteriophages to evade phage lysis is the CRISPR (clustered regularly interspaced short palindromic repeats) system (Molineux, 1991; Szczepankowska, 2012). Such bacteria possess the ability to degrade foreign DNA and create a “memory record” of this possible hostile DNA. Endonucleases, e.g. Cas9, neutralize lytic phages based on the “memory” of the CRISPR system. Why this possible resistance is not detected in vitro is debatable. Discrepancies between in vivo and in vitro conditions are possible. Resistances were also found in this study. The findings of *S*. O11 and of *Salmonella* spp. without expression of O-antigens could also be related to development of a partial resistance through mutation.

Another described bacterial defence mechanism against lytic phages are abortive infection systems (abi). The phage infected bacteria use the abi system to cause cell death before any phage can be released (Fineran et al., 2009; Speranza et al., 2011). This defence system is a further possible explanation for the variability in reduction of the various serovars.

The application of the defined *S*. Eastbourne serovar lead to effective colonization in all animals infected. This serovar colonized the gastro-intestinal tract very quickly, in one case after a single dose. The delay in the detection of this serovar in the faeces of the animals in the phage group indicates that colonization with *S*. Eastbourne was at least inhibited by the phages. Based on these results, it appears that a phage will not effectively prevent infection with a sensitive *Salmonella* serovar, but can nevertheless play a role in the colonization process.

In some animals, the application of *S*. Eastbourne seemed to have an effect on the serovars that were initially isolated, sometimes even replacing them during the course of the study. This possible interaction, and possible competition between the serovars, needs further evaluation before conclusions can be drawn.

Following gross pathology, *Salmonella*, including *S*. Eastbourne, were detected in the liver. Fiorentin et al. (2005) also detected *S*. Enteritidis in the livers of chickens following experimental infection after oral phage cocktail application, and despite significantly reduced caecal contamination. These cases of detection in the liver tissue might indicate a possible further mechanism for intermittent *Salmonella* shedding, and also explain the continuous detections of phage sensitive *Salmonella* serovars, as the bacteria could create a reservoir independent of biofilm formation, creating so-called VBNC (valuable but not cultureable bacteria). Those bacteria can be found during necropsy, but cannot be cultivated using typical microbiological procedures.

One bearded dragon was positive for *Salmonella* detection for the first time after a period of nine months. This shows that even animals that are tested negative over a perlonged period can still shed *Salmonella* later on. Reptiles should therefore be considered potential *Salmonella* carriers, even if repeated tests indicate that they are negative.

4.3 | Quantitative *Salmonella* detection

Salmonella quantification also demonstrated a great deal of variability both within individuals as well as between individual bearded dragons, making interpretation, especially of individual results, difficult. However, the significant reduction of *Salmonella* titres in the second half of Study 1 indicates that the Felix O1 phage was able to reduce the intestinal load of *Salmonella* in the treated animals. Furthermore, as already mentioned above, the phage detection rate from cloacal swabs showed some correlation with *Salmonella* shedding, as shown in Figure 1. Once the *Salmonella* titre in the faeces was reduced below a certain level, no phage was detectable. A low detection level of phages, on the other hand, was followed by an increase in the *Salmonella* titre. This could be the result of a direct effect of the phages on the *Salmonella* in the intestines.

The *Salmonella* titres measured from the animals in the phage group were reduced during the phage administration period compared to the mean shedding measured in Study 1, although the differences were not statistically significant. These three bearded dragons all tested positive for phage sensitive serovars. It is therefore likely that the Felix O1 and S16 phage cocktail was a direct cause of the decreased titres measured.

The lack of a quick significant effect of the Felix O1 phage on *Salmonella* shedding in Study 1 might be the result of the usage of a single phage. Several authors have suggested the use of phage cocktails for the effective reduction of intestinal *Salmonella* and to prevent the development of resistance (Abedon, 2017; Adhikari et al., 2017; Fiorentin et al., 2005; Lim et al., 2011). The partial effect

FIGURE 2 Bearded dragon (*Pogona vitticeps*)

of the phages used in the present study on *Salmonella* stands in contrast with study results from swine and poultry (Adhikari et al., 2017; Fiorentin et al., 2005; Lim et al., 2011; Zhang et al., 2015). For example, Gebru et al. (2010) used food additive anti-*Salmonella* Typhimurium bacteriophages in pigs and reduced *Salmonella* shedding significantly.

There are several possible explanations for the variability of the *Salmonella* titres measured, even within a single animal over a short period of time, including the quality of the faecal samples, the sample processing protocol, and the fact that *Salmonella* are capable of forming biofilms which can have a significant effect on both phage efficacy as well as *Salmonella* shedding (Speranza et al., 2011).

In this study, although only fresh samples were collected and analysed, the period of time in which the faeces were exposed to the environment and UV light did differ. The detection method used, based on colony counting, was always carried out by the same person, but the method itself does lead to some variation in the result.

Biofilms are often a community product consisting of one or several bacterial species. *Salmonella* are highly adaptive in adhering and forming biofilms on different organic and inorganic surfaces (Joseph et al., 2001; Speranza et al., 2011). Adverse environmental conditions are signals for the formation of biofilms that regulate gene expression (Gerstel & Römling, 2001; Hooton et al., 2011; Speranza et al., 2011). At the preferred temperature for *Salmonella* of 37°C, no biofilm formation was detected (Gerstel & Römling, 2001). Reptiles are poikilothermic and even though environmental temperatures reach 35°C–40°C underneath heat lamps, they are lower at night (usually between 20°C and 25°C). Reptiles and *Salmonella* are then in temperature ranges that might mediate biofilm formation.

Biofilms are also an effective defence mechanism against phages. Several factors that reduce the ability of phages to reach and lyse bacteria have been described (Abedon, 2017). In a study on the ability of phages to overcome the resilience of several *Salmonella* serovars in biofilms, the phages did inhibit biofilm formation on the three tested surfaces. However, the phage cocktail was no longer effective in controlling the biofilm after 72 hr (Garcia et al., 2017). Comeau and Krisch (2005) reported that *Salmonella* develop a resistance to phages in that time, leading to a build-up of the biofilm. Since the bacteriophages Felix O1 and S16 were studied in vivo in our study, and the phage titres never reached the extremely high titre of 10^9 pfu/ml, it is conceivable that the concentration of phages used was insufficient to significantly affect biofilms.

Therefore, conclusions with relation to the *Salmonella* quantification should be drawn carefully and only based on the average values of several samples.

4.4 | Compatibility of phage and Salmonella administration

During the phage application and the post application period, all animals were closely monitored for clinical signs, but only the faecal consistency deferred in some animals. This can be explained by a relatively higher oral intake of fluids during the time in which the animals were given the phage suspension, especially in the smaller reptiles, as this species normally does not drink much water.

The two animals from the phage group that were not euthanized and necropsied are currently still alive and in good health. This is in accordance with studies in pigs and chickens, in which no side effects were detected (Gebru et al., 2010, Hooton et al., 2011, Hong et al. 2013, Lim et al., 2012, Zhang et al., 2015). The phage cocktail consisting of Felix O1 and S16 should be considered safe for oral administration in bearded dragons. Further data on longer or different application intervals are necessary to draw conclusions on the long-term safety. (Figure 2).

The changes in *Salmonella* serovars and the use of two *Salmonella* specific phages also did not affect the health status of the bearded dragons. *S*. Eastbourne was given orally, which is a common route of infection for *Salmonella*. After detection of *S*. Eastbourne also no clinical changes were observed. This supports the theory that *Salmonella* colonize the animals without an immune reaction or pathology, as described by Monzón Moreno et al. (1995). In consequence, *Salmonella* should be considered commensal bacteria in healthy bearded dragons.

4.5 | Conclusions

The study results demonstrate that phages.

a. can be administered to reptiles without harming the animals
b. pass through and remain in the intestine for an extended period of time
c. appear to be able to replicate in *Salmonella* within the intestines and are able to influence the number and types of *Salmonella* shed

The phage application measurably reduced *Salmonella* excretion as well as the variability of the faecally shed *Salmonella* serovars. The phages did not prevent an orally administered *Salmonella* serovar from colonizing the intestines.

This study provides the first data on the potential benefits of phage administration in bearded dragons to reduce *Salmonella* shedding and related environmental contamination, and can serve as a basis for larger studies in different animal collections.

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CONFLICT OF INTEREST

The authors declare that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript. REM is employed by a commercial veterinary diagnostic laboratory. This employment did not influence study design or interpretation.

AUTHOR CONTRIBUTION

Kevin Renfert: Conceptualization; Data curation; Formal analysis; Methodology; Resources; Visualization; Writing-original draft; Writing-review & editing. **Wolfgang Rabsch:** Conceptualization; Formal analysis; Methodology; Project administration; Resources; Validation; Writing-review & editing. **Angelika Fruth:** Methodology; Validation; Visualization; Writing-review & editing. **Rachel Marschang:** Formal analysis; Writing-review & editing. **Stephanie Speck:** Investigation; Methodology; Resources. **Michael Pees:** Conceptualization; Data curation; Funding acquisition; Methodology; Project administration; Resources; Software; Supervision; Writing-review & editing.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The animal studies were performed with the approval of the responsible German authorities according to European standards (Reg.-Nr.: TVV 15/17).

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