Supplementary Information

Second-hand toxicity: A feasibility study of implant-mediated secondary poisoning in stoats

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Materials and methods

Materials

Potassium chloride (KCl, Analytical Reagent (AR) grade), sodium chloride (NaCl, AR grade), potassium dihydrogen orthophosphate anhydrous (AR grade), disodium hydrogen orthophosphate (AR grade), toluene (AR grade), and concentrated hydrochloric acid (HCl; 37%, RCI premium) were purchased from ChemSupply (Australia). Microcrystalline cellulose (MCC) NF was purchased from PCCA (USA). α-Lactose was provided by CSIRO (> 99% (NMR), Australia). Rhodamine B (≥ 95% (HPLC)) was purchased from Sigma-Aldrich (USA). Sodium fluoroacetate (1080; 89.6%) was purchased from Animal Control Technologies Australia (Australia).

Ethyl oleate (EO), and poly[(2-vinylpyridine)-*co*-(butyl methacrylate)-*co*-(isobornyl methacrylate)] with a monomer ratio of 50:30:20 mol% (PVBI), were synthesised as previously reported (Brewer et al., 2023; Brewer et al., 2022). The pH-responsive copolymer formulation PVBI/EO_{10} was prepared by combining PVBI (9 g) and EO (1 g) in toluene at a concentration of 10% w/v. Ultrapure water (≥ 18.2 MΩ·cm) was obtained from an Arium Pro Ultrapure Water System (Satorius, Goettingen, Germany).

Implant manufacture

Spherical secondary poisoning implants (SPIs) were prepared through wet granulation extrusion-spheronisation. 1080 (27.1 g), MCC (12.5 g), lactose (12.5 g), and rhodamine B (527 mg), were blended and mixed in an electronic coffee grinder (Breville). Ultrapure water (16 mL) was added to afford a damp mixture. The mixture was extruded through a 1 mm extrusion screen rotating at 35 rpm (Extruder 20, Caleva Process Solutions, UK). The extrudate was spheronised using a multi bowl spheroniser (Caleva Process Solutions, UK) at 1250 rpm for 4.5 min and then twice at 3050 rpm for 1 min, using a 250 mm diameter bowl, fitted with a 3 × 3 mm cross hatch pattern disc. Spherical particles were dried in an oven (70 °C, 14 h), then sieved to obtain a particle size fraction between 1.18–1.25 mm. This fraction formed the toxic SPI cores used for subsequent coating (**Figure S1**).

The spherical SPI cores (> 8500) were coated with the PVBI/EO_{10} coating formulation using a benchtop Mini Coater/Drier 2 (Caleva Process Solutions, UK). The formulation being spray coated was supplied to the instrument using a NE-1000 Programmable Syringe Pump (New Era Pump Systems, USA) fitted with a disposable Luer lock

syringe containing the formulation. The coating formulation was pumped at a flow rate of 15.2 mL/h with an atomising air pressure of 5 psi. The temperature-controlled fluidisation air was set to ambient temperature (22 ± 1 °C) at a fan speed of 85% (14.5 m/s). In a single coating cycle, the SPI cores were coated for a minimum of 45 min, then removed from the spray coater chamber and dried for a minimum of 15 min *in vacuo* at ambient temperature (0.1 mbar) (**Table S1** and **Figure S2**). The SPIs were coated in repeated cycles until the desired coating thickness was achieved (~ 100 and 200 µm).

Microscopy and sizing

Representative samples of the SPIs were imaged before and after coating (n = 30 and n > 100, respectively) using an Olympus SZ61 stereomicroscope fitted with a Gryphax Arktur (Jenoptik AG, Jena, Germany) camera module, using the Gryphax (v2.2.0.1234) software. To maximise the spatial resolution of the stereomicroscope, the representative samples were obtained from subsamples (n ≈ 30) of SPIs removed at random from the complete batch and spread evenly on the microscope stage (**Figure S3**). The acquired images were processed in ImageJ (v1.53g) and the *Analyze Particles* function used to determine the minimum Feret diameter of the SPIs (Igathinathane et al., 2009; Mazzoli and Favoni, 2012; Pérez et al., 2019; Rasband, 1997-2015; Vhora et al., 2024). Images acquired via the Gryphax (v2.2.0.1234) software were calibrated using an eyepiece graticule to afford an accurate scale bar. The scale bar was used to calibrate the relative pixel size in ImageJ by drawing a straight line along the scale bar using the *Straight* tool, followed by the command *Analyze → Set Scale,* and inputting the known scale distance and units. The particles to be measured were outlined using the *Rectangle* tool, and the scale bar and file information removed by selecting *Image → Crop*. The image type was changed to 8-bit (*Image → Type → 8-bit*) and made binary (*Process → Binary → Make Binary*). The particle sizes were then measured using the Analyze Particles command (*Analyze → Analyze Particles*). The size (i.e., expected size) and circularity of the particles was set to the default parameters and only changed if the imaged particles were not correctly identified by the program. Particles in close contact that were poorly resolved and not detected as single particles, were ignored. The resulting measurements in the *Results* window were exported as a .csv file (*File → Save As*) and analysed in Microsoft Excel to determine the average, standard deviation, and percentage relative standard

deviation of the minimum Feret diameter (i.e., referred to as *MinFeret* in the *Results* window).

In vitro release

The pH 1.5 and 1.7 release solutions were prepared from KCl (50 mM) solutions. HCl (pH 1.0) was used to adjust the pH values of all release solutions to within 0.02 units of the target pH using a NIST standard-calibrated pH 700 benchtop meter (Oakton Instruments, USA). Release of the SPI core contents was monitored inline using UV-visible (UV-vis) spectrophotometry, as previously described (Brewer et al., 2023; Brewer et al., 2022). Briefly, in a quartz cuvette, SPIS were suspended in 3 mL of either pH 1.5 or 1.7 release solution using a nylon mesh, under constant stirring at 37 °C. The presence of rhodamine B dye in the release solution was monitored *via* its absorbance at 543 nm (λ_{max}). In all release experiments, the initial and complete release of core contents was defined as the time at which 5 and 90% of the maximum absorbance was reached, respectively.

In vitro stability

Aliquots of phosphate-buffered saline (PBS) (10 mM, pH 7.4, 3 mL) were dispensed into plastic vials and individual SPIs (n = 10) were placed in each vial. Vials were sealed, stored at 37 °C and monitored daily. SPI polymer coating stability and failure were monitored qualitatively by assessing for the appearance of pink rhodamine B dye in the receiving solution.

In vivo ingestion trial

All procedures were approved by the Manaaki Whenua – Landcare Research Animal Ethics Committee (now the Bioeconomy Science Institute Maiangi Taiao); AEC approval number 23/10/03).

Twelve wild-caught stoats (7 male, 5 female) were obtained from professional trappers and housed at the Manaaki Whenua – Landcare Research Animal Facility, Lincoln, NZ (now the Bioeconomy Science Institute Maiangi Taiao). Stoats were fed daily with rabbit meat, chicken mince or dead day-old chickens, and had free access to water and cat biscuits. Wild-caught mice (n = 48) were housed individually in indoor enclosures with constant room temperature (20–22°C) and a natural lighting cycle. Rodent pellets (lab diet Prolab RMH 1800) and water were provided *ad libitum*, supplemented daily with fruit and seeds. Prior to insertion of SPIs, mice were

humanely killed by CO_2 inhalation. The dead mice had an SPI inserted subcutaneously, using a trocar, between the shoulder blades.

To confirm that stoats would ingest the dead mice, a pilot group of six stoats were offered a dead (non-toxic) mouse in addition to their normal maintenance diet. Stoats that had consumed a mouse were subsequently offered a dead mouse containing a SPI, and all other food was removed from the pen. The stoats were left undisturbed for 3 h before being checked to avoid interrupting feeding and were then monitored for symptoms of toxicosis every 2 h until 12 h after the implanted mouse was introduced. The following morning, the fate of each stoat (killed or not killed) was recorded, and the pen searched to confirm mouse and implant ingestion.

Initially, six stoats (3 male, 3 female) were offered dead mice with SPIs bearing a 200 µm coating. A further six stoats (4 male, 2 female) were later offered mice with SPIs that had a thinner 100 µm coating.

In vivo gastric pH testing

The gastric pH of six female stoats euthanised for tissue collection under a different project (AEC 22-11-03) was tested using pH-indicator strips pH 1-6 (pH-Fix 0-6, Fisherbrand). The six stoats were euthanised at different times over several days therefore the length of time between last food consumption and stomach pH testing varied for each animal. Gastric pH was measured following tissue collection, ~ 30 min post-mortem. A slit was cut into the stomach wall with a scalpel and the pH strip inserted. The stomach was massaged for 5 seconds, then the strip removed and pH determined from matching colour change of the strip to the pH key on the box.

Implant preparation and analysis

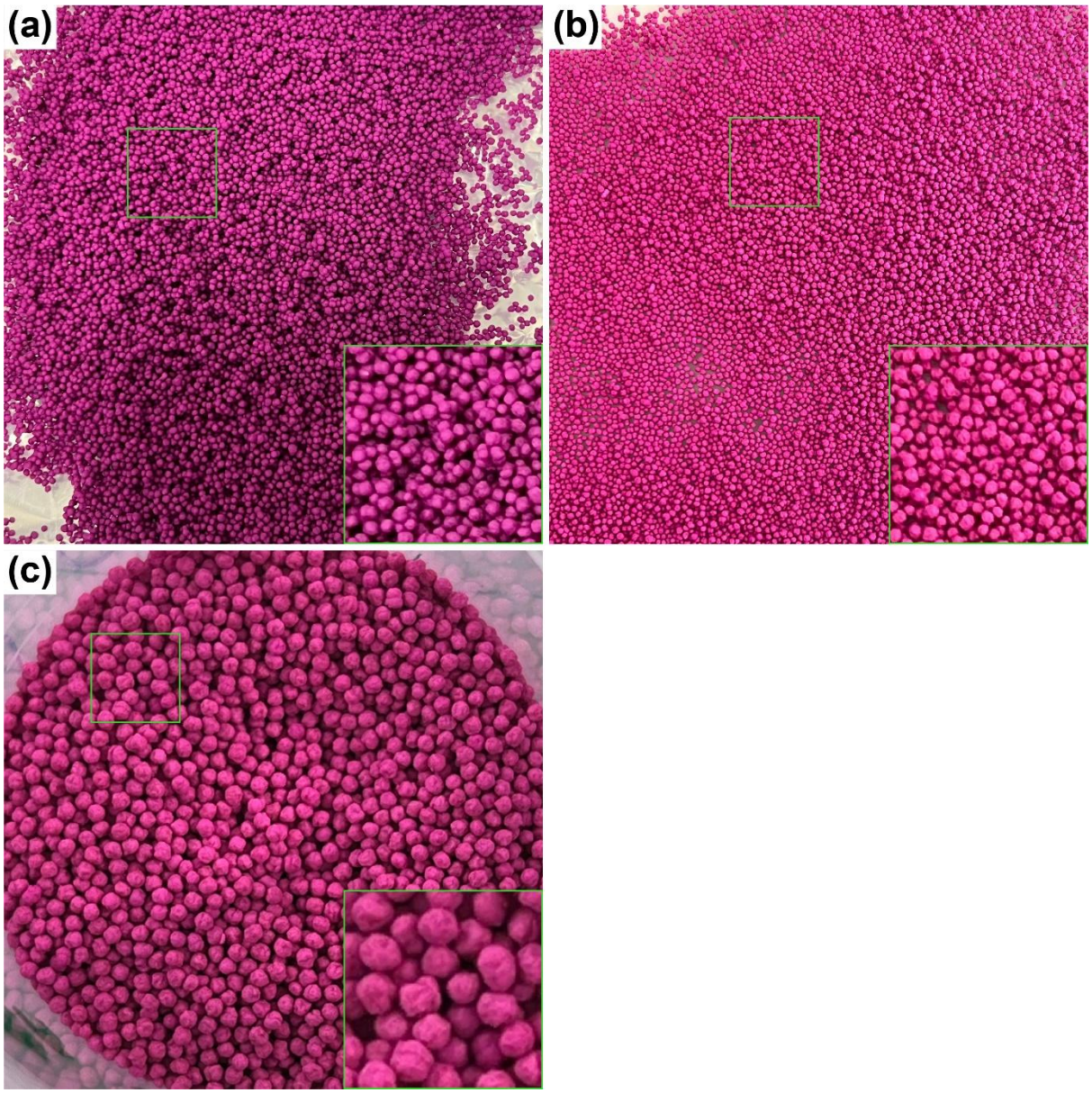

Figure S1: Images of (a) 1080-loaded spherical particles immediately following wet granulation extrusion-spheronisation, (b) after drying at 70 °C for 14 h, and (c) the final sieved particle fraction (diameter 1.18−1.25 mm) used for the SPI cores.

Table S1. Parameters for polymer spray coating onto the SPI cores, and mass gain and coating efficiency, as a function of coating cycle. Spray coater fan speed (85%, 14.5 m/s), atomizer pressure (5 PSI) and air temperature (22 ± 1 °C) were constant across all coating cycles.

Coating cycle	Duration (min)	Drying time (min)	Mass gain /SPI (mg)	Cumulative mass gain/SPI (mg)	Coating efficiency (%)
1	45	15	0.057	0.057	45.4
2	45	15	0.060	0.117	47.5
3	60	15	0.080	0.197	47.3
4	60	870	0.079	0.276	46.7
5	60	15	0.077	0.353	45.4
6	53	15	0.074	0.427	49.9
7	60	20	0.087	0.514	51.7
8	70	15	0.100	0.615	50.9
9	70	840	0.090	0.704	45.4
10	70	1080	0.095	0.799	45.2
11	70	840	0.093	0.893	44.5
12	74	20	0.118	1.010	53.2
13	82	40	0.132	1.143	53.8
14	90	90	0.141	1.284	52.4

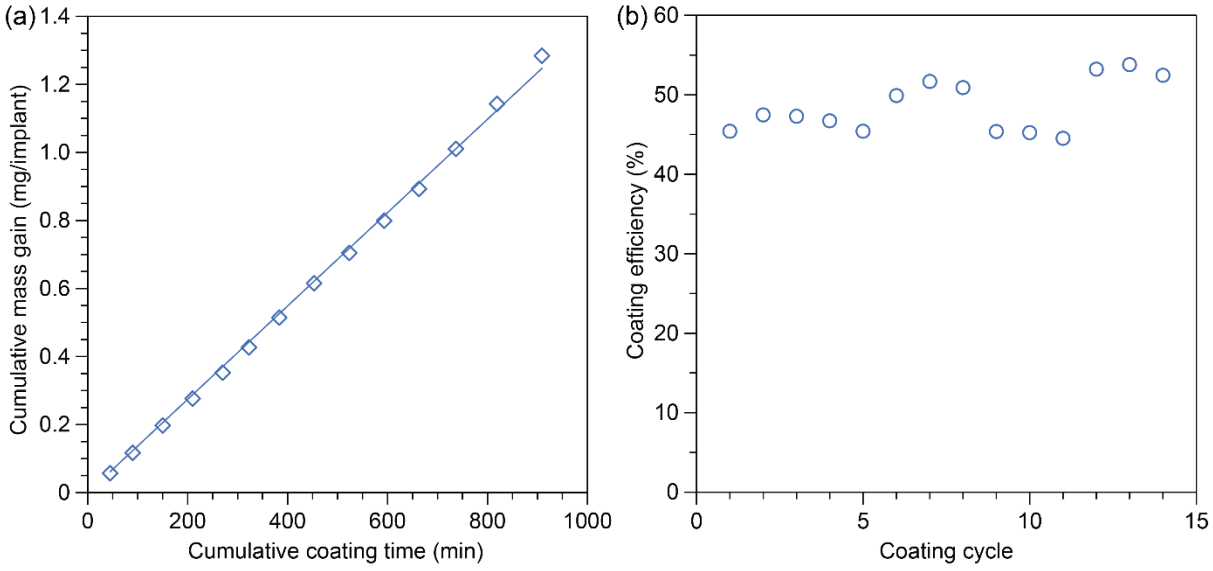

Figure S2: (a) Cumulative mass gain per SPI as a function of polymer spray coating time, and (b) coating efficiency as a function of polymer spray coating cycle.

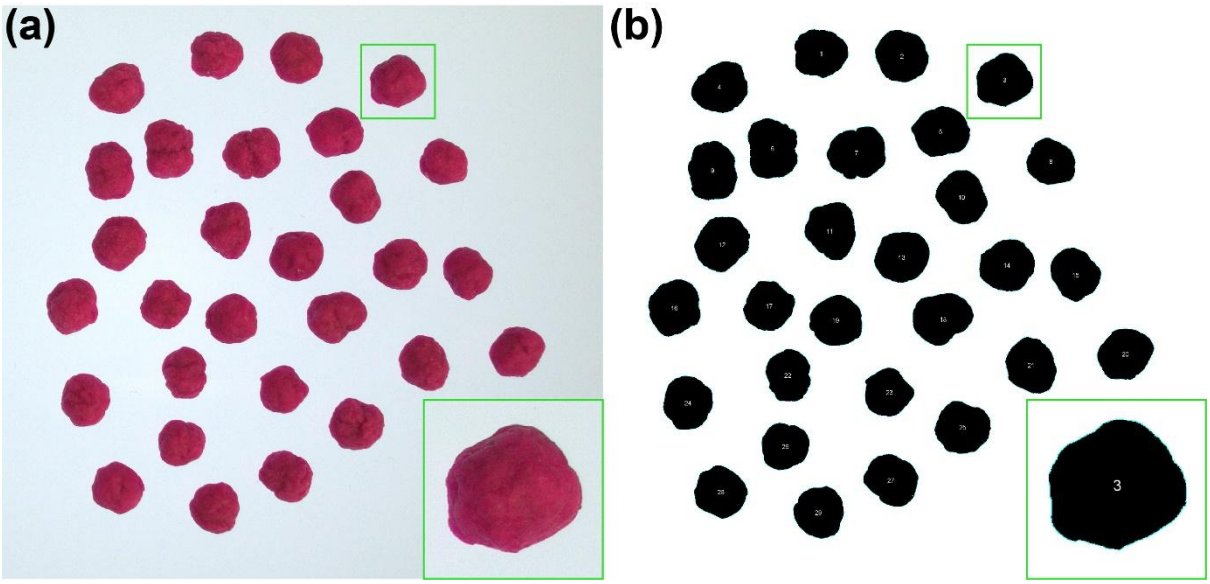

Figure S3: Representative (a) unprocessed and (b) ImageJ processed microscopy images of uncoated SPIs. Note the enlarged insets of a single uncoated SPI highlighted in green, and the outline and numbered label present on the processed image.

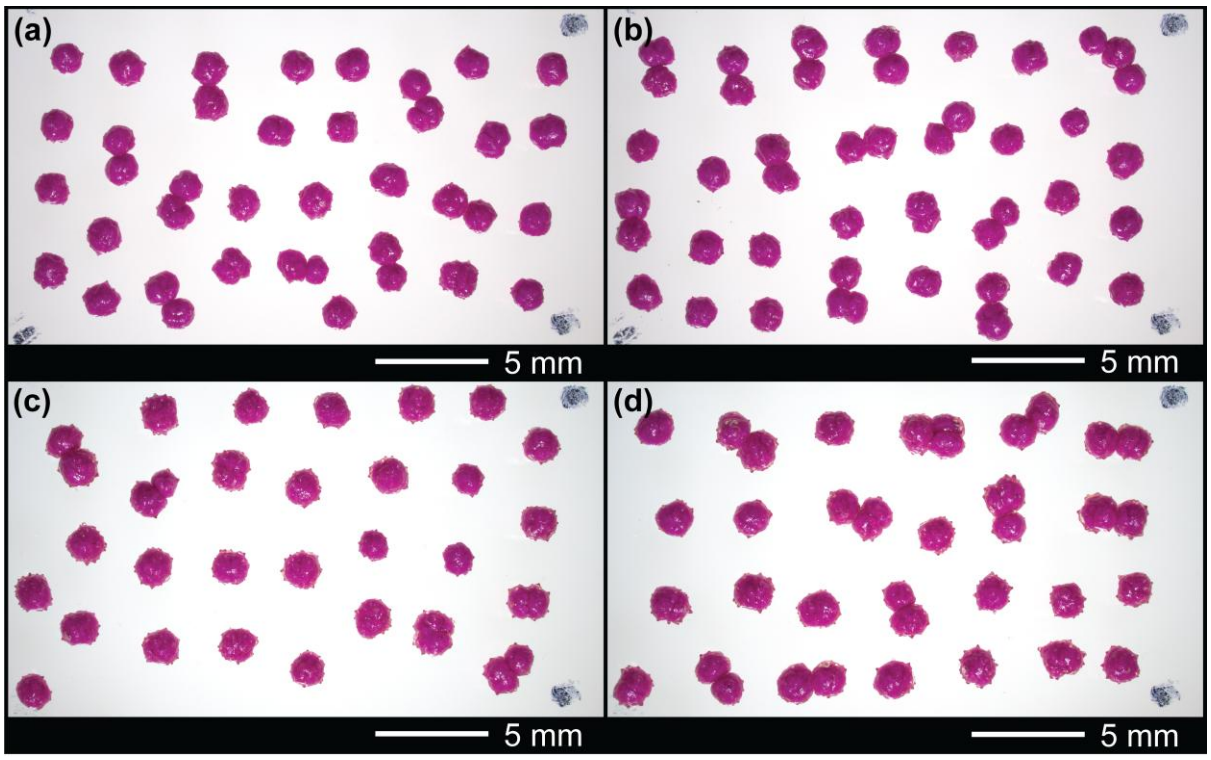

Figure S4: Images of 1080-loaded spherical SPIs after spray coating with (a,b) 100 and (c,d) 200 μm thick polymer coatings. All images show the particle twinning that occurs in some instances during spray coating.

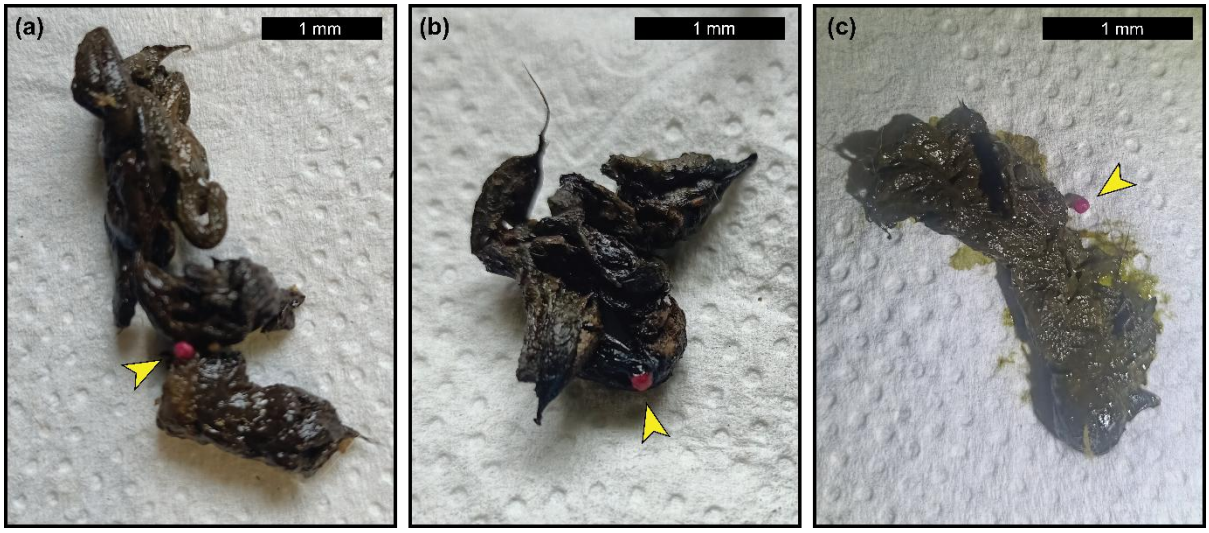

Figure S5: Images of stoat faeces complete with intact SPIs that have passed through the digestive system from the (a,b) first and (c) second trials.

References

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