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Research paper

Effect of warming and infusion of red blood cell concentrates on markers of haemolysis: An ex vivo simulation study

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ABSTRACT

Background: Transfusion of red blood cell (RBC) concentrates is a common procedure to restore blood volume and tissue oxygen delivery in patients with trauma. Although RBC warmers may prevent hypothermia, some warming or infusion equipment may lead to haemolysis and patient injury.

Objectives: The aim of this study was to test the effect of (i) RBC warming and (ii) administration via manual vs. pump infusion on haemolysis.

Methods: This experimental ex vivo study studied haemolysis markers of RBC injury. The sample consisted of 90 RBC infusions in two simulations, randomly, 45 warmed RBC infusions and 45 nonwarmed RBC infusions, in two or three stages: before the intervention (*baseline*—warming, $N=45$; nonwarming, $N=45$), after water bath warming at 42 °C (*warmed*, $N=45$), and then after the warmed or nonwarmed RBCs were infused by manual or pump infusion at a rate of 100 mL/h (*infusion*—warming, $N=45$; nonwarming, $N=45$).

Results: Warmed RBCs showed significantly lower total haemoglobin (Hb) and haematocrit levels and increase in free Hb levels, haemolysis levels, and lactate dehydrogenase (LDH) activity (all $p<0.05$) than *baseline* RBCs. Pump *infusion* RBCs were associated with reduced total Hb and increased free Hb, haemolysis, and potassium (K) levels (all $p<0.05$) compared with *warmed* RBCs. In contrast, manual *infusion* of warmed RBCs resulted in significantly reduced total Hb levels and increased LDH activity (both <0.05). After *infusion*, total Hb, free Hb, haematocrit, haemolysis, and LDH values were significantly different for warmed vs. nonwarmed RBCs ($p<0.05$).

Conclusions: Haemolysis biomarkers increase with RBC warming and infusion, especially when using infusion pumps. Critically ill patients should be carefully monitored for possible complications during and after RBC infusion.

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1. Introduction

Red blood cell (RBC) transfusion is an essential therapeutic intervention to reduce morbidity and mortality, with approximately 100 million RBC transfusions occurring worldwide each year. RBC transfusion has been highlighted by the World Health Organization as a potential risk to patient safety owing to the number and severity of related adverse events [1], such as haemolysis, caused by cell damage that may occur during donation, storage, handling, and/or administration of RBC units [2].

Haemolysis is undesirable because it reduces intact RBC delivery to the patient and increases free haemoglobin (Hb) levels in the plasma in association with clinical symptoms and outcomes including fever, chills, pain, hypotension, dyspnoea, haemoglobinuria, haemoglobinaemia, disseminated intravascular coagulation, acute renal failure, shock, and death [3,4].

Canadian [5], Brazilian [6], Australian [7], and European [8] regulations recommend that for quality control of transfusion in blood banks, the haemolysis level in RBC units must be <0.8% on the last day of maximum storage duration, which would be on the 35th d, when using an anticoagulant or preservative solution containing CPDA-1 (citrate phosphate dextrose-adenine), and on the 42nd day, with the use of other solutions for preservation [5–8]. The American Association of Blood Banks states that on the 42nd d of storage, the level of haemolysis in RBCs should not exceed 1% [9]. This is predominantly due to older RBCs being more susceptible to haemolysis [3].

Haemolysis is typically associated with markers that reflect the release of RBC contents into the aqueous solution of RBC units: total Hb (its reduction will show low content of Hb present in erythrocytes [6]), free Hb (its increase in plasma reflects destruction of RBCs and release of Hb [3,10]), potassium (K^+ ; its increase in the extracellular environment may be associated with the erythrocyte lesion [11]), and lactate dehydrogenase (LDH; its increase in enzymatic activity can occur after erythrocyte damage [12,13]).

Warming of RBCs is recommended by transfusion guidelines, but only for certain clinical situations, such as massive transfusion, wherein about half of the patient's blood volume requires rapid replacement [2,5,8,14–16]. This is especially so in trauma, which accounts for 10% of world mortality [17]. In addition, warming is recommended for adults who receive RBCs more rapidly than at a rate of 50 mL/kg/h and for children at transfusions at a rate higher than 15 mL/kg/h (assuming a child is 6.7 kg, the flow will be more than 100 mL/h) [5,8,14–17].

Warming requires raising the RBC temperature, so the patient receives blood at body temperature ($\sim 37^\circ\text{C}$), thus avoiding hypothermia ($<35^\circ\text{C}$) and its side effects. Studies have shown that 14% of mortalities occurring when transfusion is required occur when the patient's temperature is lower than 32°C [18,19]. Therefore, rapid and low-temperature infusions increase morbidity and mortality in severe and vulnerable patients, inducing cardiorespiratory arrest [14–16,20,21].

Healthcare institutions often use different methods for warming RBCs [22,23], including in-line warming using an electronic device attached to the infusion tubing [2,5,16,22–24] and immersion of the RBC bag in a warm water bath in developing countries [5–31]. However, not all practices are considered safe by the Association of Blood Banks [9] and the European Council [5].

A wide range of equipment and devices are used to administer RBCs, which also differ in the type of pressure exerted to RBCs and flow, which may contribute to haemolysis. Among the most commonly used devices in health centres are gravity-driven sets with manual flow control and electronic infusion pumps [2,33].

Thus, many questions remain about the effect of warming and infusion methods on the quality of RBC transfusions and patient

safety. Owing to the scarce and outdated literature, the aim of this study was to test the effect of warming RBC units in a water bath and subsequent infusion using manual or electronic devices on haemolysis.

The study hypotheses were that both warming and infusion pump delivery (compared with manual infusion) increase haemolysis of infused RBCs.

2. Methods

An *ex vivo* experimental study was carried out under laboratory-controlled temperature (range = 20.0°C to 25.8°C) and humidity (range = 49.0–73.0%) conditions. During the experiments, the investigators used standard precautions and appropriate disposal of blood-containing materials, and all transport and handling of RBC samples occurred as per the recommendations of the local official regulatory agency [6]. In the case of warmed RBC experiments, the use of blood with a short storage period (<10 d) was prioritised to mimic the practice in cases of massive transfusion with indication for RBC warming [2].

The sample consisted of two simulations, with 45 experiments of warmed RBCs infusions and 45 experiments of nonwarmed RBC infusions (a total of 90 infusions). The infusion methods used were randomised to one of the five infusion systems, each of which was studied nine times in each simulation: manual set drips, manual microdrips, linear peristaltic infusion pumps, syringe infusion pumps, or cassette infusion pumps.

The RBCs were separated based on the following design, and we collected specimens to analyse haemolysis markers at two (non-warming simulation) stages or three (warming simulation) stages:

- **Baseline:** RBC collection was performed directly from the RBC bag using sterile technique (warming, $N = 45$; nonwarming, $N = 45$).
- **Warmed:** RBC collection was performed directly from the RBC bag using sterile technique after warming to 42°C . This collection occurred only in the warming simulation (warming, $N = 45$).
- **Infusion:** Collection of warmed or nonwarmed RBCs was performed using sterile technique, and the RBCs were collected at the distal outlet of the device, after manual or pump infusion (warming, $N = 45$; nonwarming, $N = 45$).

Before experimentation, the stored RBC units were kept in CPDA-1 anticoagulant preservative solution, in polyvinyl chloride bags, and refrigerated at $2\text{--}6^\circ\text{C}$. The blood bag was removed, kept at room temperature for 30 minutes, and manually mixed; the connection point was disinfected with 70% alcohol, infusion tubing was inserted into the bag, and the collection at the *baseline* stage was performed directly from the RBC unit, in both simulations.

In the simulations of warmed RBC infusions, the RBC units were immersed in a water bath (Water Bath, Fanem®, model 1102, Guarulhos, SP, Brazil), with temperature controlled at 42°C using a visible thermometer. For immersion, RBC units were wrapped in a plastic protector to prevent contamination [24]. The RBC bag was removed and gently mixed every 10 minutes for temperature verification using an infrared thermometer until reaching 42°C , after 30 minutes, on average. The choice of the temperature of 42°C for the experiment aimed to represent the maximum level allowed as per studies and guides of conduct [5,9,22,23]. The collection of RBCs from warmed units for analysis of the *warmed* stage was carried out directly from the bags (in the same way as for the *baseline* stage), immediately after reaching 42°C , approximately at 30 minutes.

After the *warmed* stage (warming simulation) or *baseline* stage (nonwarming simulation), RBC units were then distributed into infusion systems using burettes containing 180- μ m filter, with the same infusion flow being determined, establishing 100 mL/h in the infusion pumps and 33 drops/min or 100 microdrips/min in the manual infusions. In each simulation, the infusion pumps were studied in triplicate (three infusion pumps from the same manufacturer) and submitted to three experimental infusions, controlling for variations in equipment. Therefore, nine analyses of infusion simulations in each infusion pump type were conducted, and the same number of simulations was performed with the manual system. The RBC collection from the *infusion* stage was performed after discarding 1.5 times the internal volume of each infusion system to avoid interference from the free-flow fill.

It is worth mentioning that the RBC bags could be used in more than one infusion method, depending on the volume and the experiments that were occurring in parallel in the laboratory; thus, these experiments were conducted using blood from 54 units in total.

In each of the previously described stages, the samples were taken to measure haemolysis markers. For analysis of total Hb and haematocrit (Hct) levels, blood was collected in a glass capillary tube (without the anticoagulant). For analysis of free Hb and K^+ levels and LDH activity in plasma, the blood was collected in a clot activator and separator gel (BD Vacutainer®, SST II Advance, Ref 367955, Plymouth, UK) tube for centrifugation (Kasvi®, model K14-4000, Parana, Brazil) at 3600 rpm for 10 minute to obtain serum [6,8,11–13,34,35]. For the level of haemolysis, the calculation was based on the identified values of free Hb, total Hb, and Hct [34,35].

Analytical equipment was maintained and calibrated before use by trained laboratory technicians, with a small fluctuation test–retest reliability considered acceptable and within the manufacturer's specifications.

2.1. Measurements

Samples were analysed as per the relevant literature [6,8,11–13,34,35].

Hct (%) identifies the proportional volume of RBCs within a sample; the expected reference range in a unit of RBCs preserved with CPDA-1 is 65–80% [6]. This analysis involved using a haematocrit microcentrifuge (MH, Celm®, Sao Paulo, Brazil) and capillary tubes filled to two-thirds of their capacity, followed by centrifuging at 11,500 rpm for 4 minutes [34].

The expected level of total Hb (g/dL) is a minimum of 45 g per bag of RBCs at the end of processing [6]. This measurement was performed by conversion of Hb into cyanomethaemoglobin, and lysis was performed by oxidation with the use of RBCs contained in the glass capillary tube. A standard Hb solution with a haemoglobin cyanide concentration of 12–15 g/dL was obtained. The reagent containing 0.1 M potassium phosphate monobasic, 0.06 M potassium ferrocyanide, 0.077 M potassium cyanide, and 0.082 M Triton X-100 (Doles®, Goias, Brazil) was used at a volume of 5 mL for each 20 μ L of RBCs. The mixture was read using a spectrophotometer at 540 nm (SP-22, Biospectro®, Parana, Brazil) [34].

The expected values of plasma-free Hb (g/dL) are between 0.007 and 0.65 g/dL in RBC units stored in CPDA-1 [3,36]. For this analysis, the final result was obtained by mixing 125 μ L of serum and 2.5 mL of deionised water (dilution: 1:20), with absorbance measured at 370, 415, 510, 577, and 600 nm, using a spectrophotometer [34].

The K^+ concentration (mmol/L) was analysed in serum after centrifugation, with 1000 μ L of 0.3 M trichloroacetic acid added to 100 μ L of serum to precipitate proteins. This solution was centrifuged again for 6 minutes at 3200 rpm. Then, 200 μ L of the supernatant was added to 2 mL of the potassium reactant for 10-

minute reset (3.2 mL of 0.18 M sodium tetraphenylborate and 3.2 mL of 2 M sodium hydroxide; both Doles®, Goias, Brazil) and was subjected to spectrophotometer reading at 580 nm [8,11].

LDH activity in serum (U/L) was assessed after centrifugation using a kinetic method with a combination of reagents (R1: 20 mL of 50 mmol/L of phosphate buffer, pH of 7.5, and 0.60 mmol/L of pyruvate; R2: 5 mL of Good's buffer, pH 9.6, and 0.18 mmol/L of NADH), both obtained from Diasys®, Rio de Janeiro, Brazil. The assay was performed by combining 2 mL of the reagent and 40 μ L of serum, with absorbance recorded over 4 minutes by spectrophotometry at 340 nm [12,13].

Subsequently, the level of haemolysis was calculated as per the following formula: [serum free Hb (g/dL) \times 100-Hct (%)]/total Hb (g/dL) [34].

2.2. Data analysis

Outcomes were described using mean (M), standard deviation (SD), minimum, maximum, and absolute difference. The Anderson–Darling normality test was applied to determine normality of data [37,38]. Parametric (paired t-test) and nonparametric tests (Mann–Whitney test) were used [37,39] to compare the markers of haemolysis of RBCs at the *baseline* and *warmed* stages and also between different infusion techniques, manual and electronic, for warmed RBCs, and to compare the effect of infusion (any method) between *baseline* and *infusion* RBCs (warming simulation) or between *baseline* and *infusion* RBCs (nonwarming simulation). The analysis software used was R 3.1.2 [40], Vienna, Austria and the level of significance was $p \leq 0.05$.

2.3. Ethical statement

The study was approved by the Research Ethics Committee of the Federal University of Sao Paulo (Unifesp), Brazil (2012/56518).

3. Results

The RBCs used in this study had blood types including A (84; 93.3%) and O (6; 6.7%) positive, with a volume per unit of 204.0–350.0 mL (M \pm SD of 265.1 \pm 41.7 mL). RBCs had variable storage times (13.0 \pm 8.2 d), of which 51 (56.7%) were <10 d, 23 (25.5%) were 11–20 d, and 16 (17.8%) were 21–35 d.

Table 1 shows that the RBCs in the *warmed* stage showed significantly lower total Hb and Hct levels and increase in free Hb values, haemolysis levels, and LDH activity (all $p < 0.05$) compared with *baseline* RBCs.

The level of haemolysis in *baseline* (warming simulation) RBCs was 0.05%, increasing to 0.13% after warming. This remained at 0.13% after manual *infusion* and increased to 0.16% after pump *infusion* (Tables 1 and 2).

In comparison with *warmed* values, Table 2 shows that RBC administration by pump *infusion* was associated with reduced total Hb and increased free Hb, haemolysis, and K^+ levels (all $p < 0.05$). In contrast, manual *infusion* significantly reduced total Hb and increased LDH values (both < 0.05).

Table 3 compares the effect of the RBC infusion (any method) at the *baseline* stage (for the warming vs. nonwarming simulation). Negative and positive values represent a relative decrease and increase in the marker, respectively. The mean changes in RBC values after the infusion of the two simulations were significantly different for total Hb, free Hb, Hct, haemolysis, and LDH ($p < 0.05$, Table 3). K^+ concentration changes did not differ significantly for the warming/nonwarming simulations.

Table 1
Comparison of haemolysis outcomes for the *baseline* (n = 45) and *warmed* (n = 45) RBCs.

Haemolysis markers	<i>Baseline</i> , M ± SD	<i>Warmed</i> , M ± SD	p (Mann–Whitney test)
Total Hb (g/dL)	26.9 ± 5.38	24.8 ± 5.72	0.002
Free Hb (g/dL)	0.06 ± 0.11	0.13 ± 0.13	<0.001
Hct (%)	73.3 ± 5.28	72.6 ± 5.44	<0.001
Haemolysis (%)	0.05 ± 0.06	0.13 ± 0.09	<0.001
K (mmol/L)	36.3 ± 12.7	34.1 ± 12.6	0.052
LDH (U/L)	409 ± 262	518 ± 268	<0.001

RBCs = red blood cells; Hb = haemoglobin; Hct = haematocrit; K = potassium; LDH = lactate dehydrogenase; M = mean; SD = standard deviation.

4. Discussion

The main finding of this study is that RBC warming using a water bath at 42 °C increases haemolysis levels, plasma-free Hb levels, and LDH activity and decreases total Hb and Hct levels. Thereafter, manual infusion of warmed RBCs significantly influenced two markers: increasing LDH activity and reducing total Hb content, without affecting haemolysis. Pump infusions of warmed RBCs were more likely to cause haemolysis than manual infusions, with significantly more haemolysis, free Hb, and K⁺ levels and a reduction in total Hb levels.

The variation in haemolysis after infusion of warmed RBCs was statistically higher than after infusion of nonwarmed RBCs, except for K⁺, which may have been influenced by the high temperature (returning into the erythrocytes) [41]. Therefore, these data suggest damage that occurred to erythrocytes owing to warming, with additional injury occurring during infusion.

Although studies [27,42] support the use of a temperature-controlled water bath to warm RBC units, our study has identified that erythrocyte injury occurs. Whether this has clinical significance will depend on the patient's condition. The majority of guidelines related to RBC warming do not recommend

the water bath method, stating that it can increase haemolysis (~30 minutes) and has potential for microbial contamination [5,8,22,34,43]. However, this is currently the method followed in developing countries owing to its low cost [25–31].

Two recent studies performed infusion with in-line warmers, with countercurrent heat exchange mechanisms, and however did not demonstrate an increase in free Hb and haemolysis levels, when temperature was adjusted to 41.5 ± 0.5 °C [21,23]. However, another study showed that in-line warming devices formed bubbles that can be fatal in infants and children [44].

Currently, the most described method of warming RBCs in the literature is in-line warming with alarms and temperature control [2,5,7–9,16,22–24,34], but owing to different warming technologies [16,45], additional studies are needed to prove the safety of such equipment for blood transfusion. As noted, in-line warming may not be available in developing countries owing to its high cost.

In our study, we demonstrated a M ± SD variation in haemolysis at the *baseline* stage in warming simulation (0.05 ± 0.06%), of 160% after warming (0.13 ± 0.09%), and of an additional 23% after infusion using infusion pumps (0.16 ± 0.10%), an increase of more than three times the original level of haemolysis. However, there was no significant increase in the mean haemolysis level after infusion using manual devices (0.13 ± 0.07%).

Similar results were reported in a study of RBCs warmed by in-line devices at 41.5 °C, whereby manual infusion using a syringe (gravitational) did not generate haemolysis, whereas infusion pumps generated 3–4 times more haemolysis, although these values did not exceed internationally recommended limits [23].

The present study is the first to study the warming of RBCs in a water bath in combination with manual and pump infusion devices. To the best of our knowledge, the last experimental study investigating the water bath warming method occurred 15 y ago; however, it used whole blood rather than RBCs [25]. Recently, two experimental studies and one systematic review conducted in Canada (2015–2016) addressed the issue of warming RBCs but did

Table 2
Comparison of haemolysis markers levels in *warmed* RBCs, before vs. after infusion by electronic (pump) and manual devices.

Haemolysis markers	Before	After pump infusion	Before vs. after pump infusion	After manual infusion	Before vs. after manual infusion
	M ± SD	M ± SD	P	M ± SD	p
Total Hb (g/dL)	24.8 ± 5.72	20.4 ± 4.83	0.003 ^a	27.2 ± 2.87	0.038 ^a
Free Hb (g/dL)	0.13 ± 0.13	0.12 ± 0.12	<0.001 ^a	0.16 ± 0.12	0.265 ^a
Hct (%)	72.6 ± 5.44	70.6 ± 5.22	0.999 ^b	75.7 ± 5.00	0.352 ^a
Haemolysis (%)	0.13 ± 0.09	0.16 ± 0.10	0.001 ^a	0.13 ± 0.07	0.246 ^a
K (mmol/L)	34.1 ± 12.60	30.1 ± 11.5	<0.001 ^a	44.5 ± 6.10	0.325 ^a
LDH (U/L)	518 ± 268	517 ± 294	0.407 ^a	674 ± 394	0.009 ^a

RBCs = red blood cells; Hb = haemoglobin; Hct = haematocrit; K = potassium; LDH = lactate dehydrogenase; M = mean; SD = standard deviation.

^a Mann–Whitney test.

^b Paired t-test.

Table 3
Comparative analysis of the absolute difference in haemolysis markers in *baseline vs. infusion* RBCs (warming simulation) and *baseline vs. infusion* RBCs (nonwarming simulation).

Haemolysis markers	RBCs, <i>baseline vs. after infusion</i> (warming simulation)	RBCs, <i>baseline vs. after infusion</i> (nonwarming simulation)	p (Mann–Whitney test)
	M ± SD	M ± SD	
Total Hb (g/dL)	–3.79 ± 4.31	–0.73 ± 4.56	<0.001
Free Hb (g/dL)	0.07 ± 0.09	0.02 ± 0.03	<0.001
Hct (%)	–0.64 ± 1.37	0.13 ± 2.02	0.038
Haemolysis (%)	0.10 ± 0.08	0.02 ± 0.03	<0.001
K (mmol/L)	–0.47 ± 5.68	0.87 ± 3.03	0.349
LDH (U/L)	170.71 ± 195.10	–21.29 ± 481.63	<0.001

RBCs = red blood cells; Hb = haemoglobin; Hct = haematocrit; K = potassium; LDH = lactate dehydrogenase; M = mean; SD = standard deviation.

not include the water bath method [21,23,46]. In addition, no previous experiments were found with short RBC unit storage times (<10 d) and this study has a diverse range of storage times in tests with warmed and nonwarmed RBCs.

The levels of haemolysis found in this study did not exceed the internationally recommended limits; however, it should be noted that many of our units were <10 d old, whereas the recommended storage of RBC units may reach 35–42 d [5,7–9,34,47]. Therefore, the acceptability of a threefold increase in the level of haemolysis in units that had a storage time of 7.0 ± 4.5 d is not generalisable to RBCs with longer storage times because this potentiates erythrocytes to have reduced deformability, enhanced fragility, and higher susceptibility to degradation by white blood cells [3]. After the warming or infusion of older RBCs, this could potentially result in transfused RBC units exceeding haemolysis limits, increasing complications in patients [5,7–9,21,23,34,45–48].

The limitations of this study included smaller subgroups of warmed and nonwarmed RBCs investigated for each type of the five infusion devices, which precludes statistical comparison of each device.

The clinical impact of these results is especially relevant in emergency or major surgical procedures for patients with indication of transfusion of warmed RBCs to avoid hypothermia, as in massive transfusion, because hypothermia aggravates morbidity and mortality of patients with severe trauma [7]. Changes in haemolysis markers, after warming in a 42 °C water bath and infusing warmed RBCs, could exacerbate or lead to clinical complications for many patients, depending on their clinical condition [2,16,36,41,44–49]. In addition, high-volume transfusions in neonates are always warmed, and in this case, even slight variations in the level of haemolysis could result in clinically significant repercussions [2,49].

We conducted this study to investigate the effect of using simpler and less costly technologies for warming of RBCs before administration and to highlight the haematological and biochemical changes of RBCs, the impairment of blood cells, and the presence of haemolysis, which may lead to a greater risk of significant clinical complications, especially in children or adults who have increased vulnerability [2].

In conclusion, although haemolysis levels did not exceed the levels recommended by blood banks from a clinical perspective, this study suggests that water bath warming (~42 °C) may not be a safe method to warm RBCs because levels of haemolysis and its markers increased after warming and were aggravated with infusion, especially via infusion pumps, despite the RBC units tested after a short storage time (<10 d). It is important for future studies to compare alternative methods of warming and infusing RBCs, evaluate appropriate warming of RBCs stored with other preservatives/additive solutions, and document current practices of RBC warming in hospitals. This will improve understanding of the risks and benefits for patients and facilitate safe RBC transfusion practices that maintain patient safety.

Conflict of Interest

Griffith University has received unrestricted investigator-initiated research or educational grants on A.J.U's behalf from product manufacturers (3M, Becton Dickinson, Cardinal Health). Griffith University has received unrestricted investigator-initiated research or educational grants on C.M.R.'s behalf from product manufacturers (3M, BD-Bard, Cardinal Health). Griffith University has received consultancy payments on C.M.R.'s behalf from manufacturers (3M, B. Braun, BD-Bard). Griffith University has received

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CRediT authorship contribution statement

Maria Paula O. Pires: Conceptualisation, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Visualisation, Writing - original draft, Writing - review & editing. **Maria Angélica Sorgini Peterlini:** Conceptualisation, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Writing - original draft, Writing - review & editing. **Amanda J. Ullman:** Data curation, Formal analysis, Resources, Writing - original draft, Writing - review & editing. **Andrew Cameron Bulmer:** Data curation, Formal analysis, Methodology, Resources, Writing - original draft, Writing - review & editing. **Claire M. Rickard:** Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Resources, Writing - original draft, Writing - review & editing. **Mavilde L. G. Pedreira:** Conceptualisation, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Resources, Visualisation, Writing - original draft, Writing - review & editing.

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