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Intermittent tensile strain induces an increased response in bone formation markers compared to continuous load in mouse pre-osteoblasts when loading magnitude is matched

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ABSTRACT

Intermittent and continuous mechanical loads are known to influence osteogenic activity. The present study examines the effects of matched intermittent and continuous load *in vitro* on bone formation markers. MC3T3 (mouse pre-osteoblasts) were cultured and placed in a bioreactor to undergo continuous, intermittent, or unloading for 1, 3 and 12 days. Loading conditions were matched for magnitude, duration and frequency. Each time point was analysed for alkaline phosphatase (ALP) activity, procollagen 1 N-terminal propeptide (PINP) and alizarin red staining (ARS). Intermittent load caused an increase in ALP activity across all time points compared to continuous loading (↑30%–59%) and unloaded conditions (↑70%–90%). PINP concentrations from intermittent load were lower than continuous load (↓112%) on day 3. However, no differences were observed in PINP concentrations between loading conditions at other time points. No differences were observed for ARS between loading conditions. Intermittent load caused an increase in bone formation marker ALP, but not PINP, when compared to continuous loading and unloaded conditions. These findings further our knowledge in bone formation response and provide additional tools for the analysis of osteogenesis *in vitro*.

1. Introduction

The mode in which load is applied can affect the bone formation response. Controlling the magnitude, frequency, duration, and type of load with a bioreactor allows conditions to be manipulated on cellular models. *In vitro*, bioreactors have been used to impose different types of load on cellular models (Zhong et al., 2013; Plunkett et al., 2010; Murray and Rushton, 1990).

For example, the application of tensile load promotes osteoblast quantity and functionality important for bone formation, whereas, compressional load has been observed to decrease the OPG/RANKL ratio and promote osteoclastogenesis (Zhong et al., 2013). Whilst compression and fluid shear stress are commonly experienced *in vivo*, tensile stress remains a common and easy method to trigger the cell response *in vitro* (Baudequin et al., 2019). *In vitro* studies remove the systemic response that is present in human studies and may help to understand the effects of load which will enable the development of *in vivo* protocols that can optimise bone formation. At present, the optimal loading regimen to promote osteogenesis, in terms of load magnitude, frequency, duration and type, remains unclear.

Animal models have demonstrated that periods of rest incorporated into cyclic loading cycles can enhance the relative bone formation rate ~2-fold compared to continuously applied cyclic loads (Burr et al., 2002). Furthermore, periosteal bone formation (Srinivasan et al., 2002), areal bone mineral density and bone mineral content (Robling et al., 2001) have been shown to increase in rodents when implementing rest periods between loading cycles. The relative mineralising surface, defined as the size of the interface between quiescent and newly formed bone, has been shown to increase after having 14 s of rest between loading bouts in comparison to 0.5, 3.5 and 7 s (Burr et al., 2002) while mechanosensitivity fully restores after 8 h of rest (Robling et al., 2001). Interestingly,George et al. (2022) compared continuous and

Abbreviations: PINP, Procollagen I N-terminal propeptide; ALP, Alkaline phosphatase; ARS, Alizarin red staining.

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intermittent loading in experimental and theoretical models. They observed intermittent load, in rats, increased cortical thickness, whereas continuous load decreased cortical thickness. A similar response was shown in their theoretical model where intermittent load favoured bone formation whilst continuous load promoted bone resorption (George et al., 2022). Rest periods are thought to resensitise bone to the effective mechanical load, which if implemented over time would likely translate into a greater osteogenic stimulus and promotion of bone formation. A major limitation of existing models is that these approaches do not match absolute loading between conditions. Failure to do this exposes cells to different accumulated loads, even when the magnitude and frequency of loading are matched, meaning the model is experiencing a different total load (LaMothe and Zernicke, 2004).

Exercise bouts consisting of intermittent loading may have a role in optimising bone mechanosensitivity. It is not clear how the method of loading affects osteoblast activity when loading magnitude and duration are matched. Therefore, this study aimed to assess the pre-osteoblast response to cyclic intermittent and continuous loading patterns. It was hypothesised that intermittent load will induce a heightened response in bone formation markers (ALP and PINP) compared to continuous load.

2. Methods

2.1. Cell culture

Mouse pre-osteoblast cells (MC3T3, ATCC) were cultured in complete growth medium (GM) composed of Minimum Essential medium α (MEMα, Gibco) supplemented with 10% foetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin solution (Invitrogen). Cells were seeded on a 6-well plate at a density of 15,000 viable cells/cm^2 and incubated in a humidified 5% CO_2 atmosphere at 37 °C. Once the cells reached 80% confluence, the GM was discarded, and the cells were washed with phosphate-buffered saline (PBS, Sigma). Differentiation medium (DM) consisting of αMEM, 10% FBS, 1% penicillin-streptomycin solution, ascorbic acid and β-glycerophosphate was then added. DM was changed every 2 days. The total culture period was 24h in GM followed by 12 days in DM. Cells were collected for analysis on days 1, 3 and 12.

2.2. Mechanical loading

The computer-controlled bioreactor (Flexcell Int. USA) was employed to deliver mechanical loading. After switching to DM, MC3T3 cells on a collagen-coated flexible bottom 6-well plate (Biopress, Flexcell) were transferred to the bioreactor to undergo cyclic loading conditions under continuous tensile strain (n = 3) or intermittent tensile strain (n = 3) (Fig. 1). Loading conditions were matched for the duration under strain (5 h), magnitude of strain (5000 μS = 0.5% elongation) and frequency (1 Hz). The magnitude of the strain was selected based on previous studies that state 5000 μS (0.5% elongation) is the upper end of physiological strain that allows for a window of optimal bone formation (Baudequin et al., 2019). For continuous conditions, 5 h of strain were followed by 19 h of rest. Intermittent loading consisted of 1 h of loading followed by 3 h and 48 min of rest every 24 h. The unloaded condition was used as a control. Cells were collected on days 1, 3 and 12 to perform ARS, ALP and PINP analysis.

2.3. ALP activity

The assay (interassay CV, 4–7%; Alkaline Phosphatase Assay kit (Colorimetric), Biovision, Abcam) was performed using multiple standards prepared of assay buffer and pNPP diluted between 0 and 50 μL. Enzyme solution was added to each well before being incubated at 25 °C for 60 min protected from light. Stop solution was added to each well after the incubation period to conclude the reaction. Post incubation the plate was gently agitated on a plate shaker and measured at an optical density of 405 nm on a plate reader. ALP activity was calculated as:

$$ALP\ Activity = \left(\frac{B}{\therefore T * V}\right) * D$$

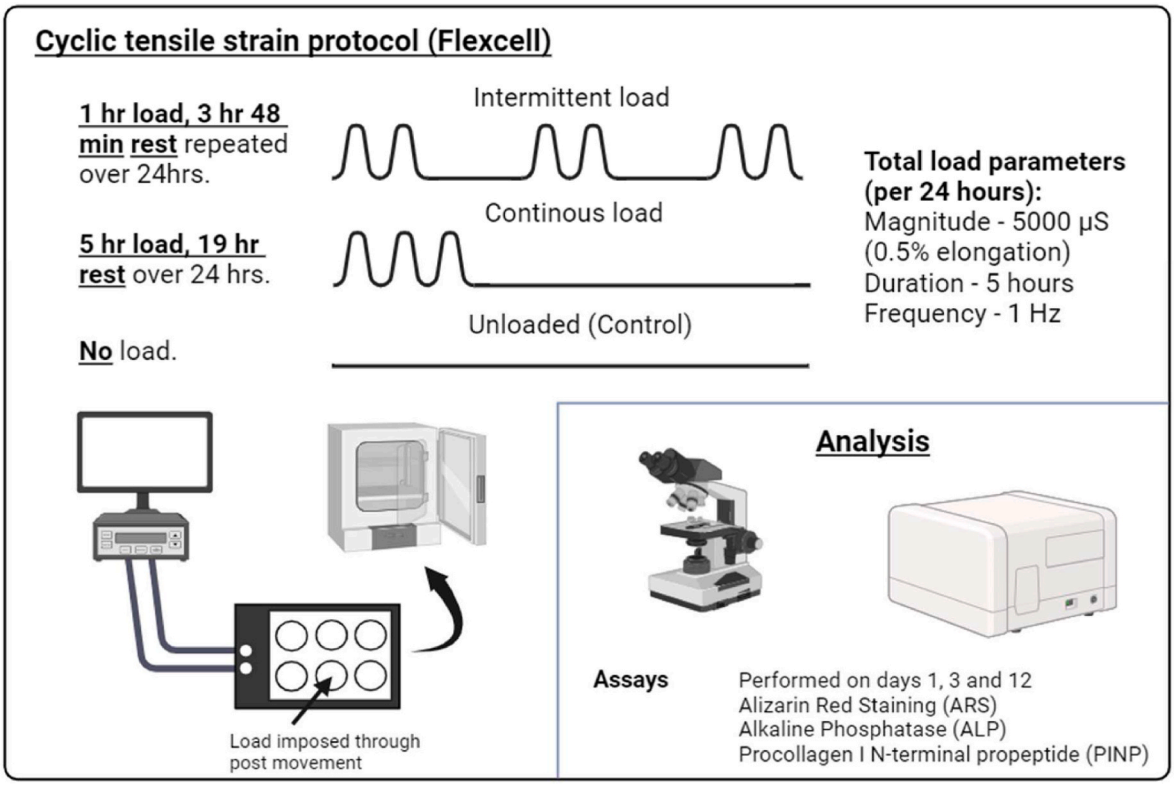

Fig. 1. Schematic of methodological processes required during *in vitro* study (created with Biorender.com).

where: B = amount of *p*NP in a well calculated from a standard curve (μmol). ΔT = reaction time (minutes). V = original sample volume added into the reaction well (mL). D = sample dilution factor.

2.4. PINP assay

The assay (interassay CV, 9–15%; IDS, Immunodiagnostic Systems) was performed using a competitive enzyme-linked immunosorbent where 50 μl of each calibrator, control and diluted sample were incubated with a biotinylated PINP reagent in microtiter wells. Rinsing with DH_2O and wash buffer was followed by the addition of enzyme avidin to the wells before another rinse was performed. Colour was developed using a chromogenic substrate (TMB). The absorbance of the completed reaction was assessed from a microtiter plate reader at an absorbance rate of 450 nm and a reference of 650 nm within 30 min of completing the reaction. The intensity of the developed colour was inversely proportional to the concentration of PINP in the original sample.

2.5. ARS staining

Alizarin red staining (Sigma-Aldrich) was performed to determine the presence of extracellular matrix mineralisation. Prior to the fixation of the cells, the DM was aspirated and transferred into a falcon tube and stored at −20 °C for further quantification analysis. On days 1, 3 and 12, cells were fixed in 10% formalin and stained with alizarin red stating solution (pH 4.0) at room temperature and protected from light for 45 min 10% acetic acid was used to collect cells (x ml/well) followed by 10min incubation at 80 °C water bath and centrifugation at 17,000g for 15min using microcentrifuge tubes. To neutralise the acetic acid, 10% ammonium hydroxide was added to the supernatant. The liquid was then aliquoted to a 96-well plate and optical density was read on a spectrophotometer at a wavelength of 405 nm. Three independent experiments were performed with duplicates for each of the 6 wells (Fig. 2).

2.6. Statistical analysis

Data were checked for normality of distribution with Shapiro-Wilks tests (IBM, SPSS Statistics, v.28). A two-way repeated measures ANOVA compared group differences between loading condition and time. To compare within-group differences a one-way repeated measures ANOVA was performed on loading conditions for ALP activity, PINP and ARS at each time point and Tukey's posthoc analysis was applied. Kruskal-Wallis tests were used if data were non-parametric. Statistical significance was accepted at the 95% confidence level ($P < .05$). Means are expressed as M.

3. Results

3.1. Alkaline phosphatase activity

There was a significant difference in timepoint ($P < .001$) and loading condition ($P = .004$) between groups in ALP activity. ALP activity was greater in the intermittent loading condition compared to the continuous loading condition on day 1 (M Intload .390 Conload .299, +30%, 95% CI: .007–.174, $P = .035$), day 3 (M Intload .404 Conload .253, +59%, 95% CI: .123–.178, $P < .001$) and day 12 (M Intload .440 Conload .313, +40%, 95% CI: .056–.199, $P = .004$; Fig. 3a). ALP concentrations were greater in the intermittent loading condition compared to the unloaded condition on day 1 (M Unload .205, +90%, 95% CI: .101–.268, $P = .001$), day 3 (M Unload .221, 82%, 95% CI: .155–.210, $P < .001$) and day 12 (M Unload .258, +70%, 95% CI: .111–.254, $P < .001$; Fig. 3a).

3.2. PINP assay

There was a significant difference in timepoint ($p < .001$) between groups in PINP concentrations. PINP concentrations were greater in the continuous loading condition compared to the intermittent loading (M Conload 66 Intload 31, +112%, 95% CI: 12–56, $P = .007$) on day 3 (Fig. 3b).

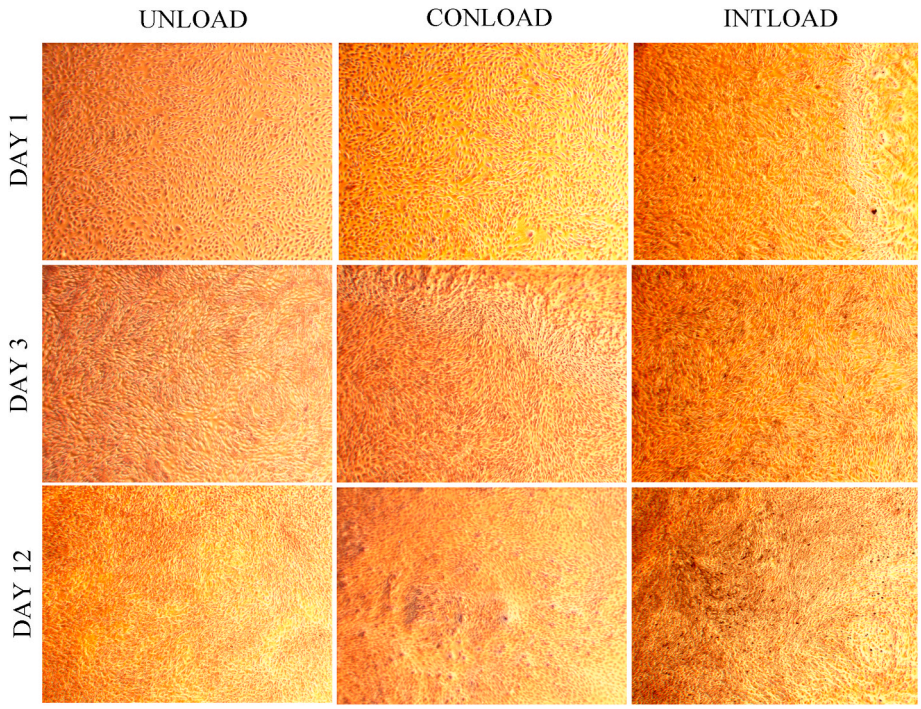

Fig. 2. Raw images of osteoblasts (x4). Qualitative alizarin red staining at days 1, 3 and 12 for each loading condition. Darker red areas correspond to calcium-rich deposits (mineralisation). Unload = control, Conload = continuous load, Intload = intermittent load.

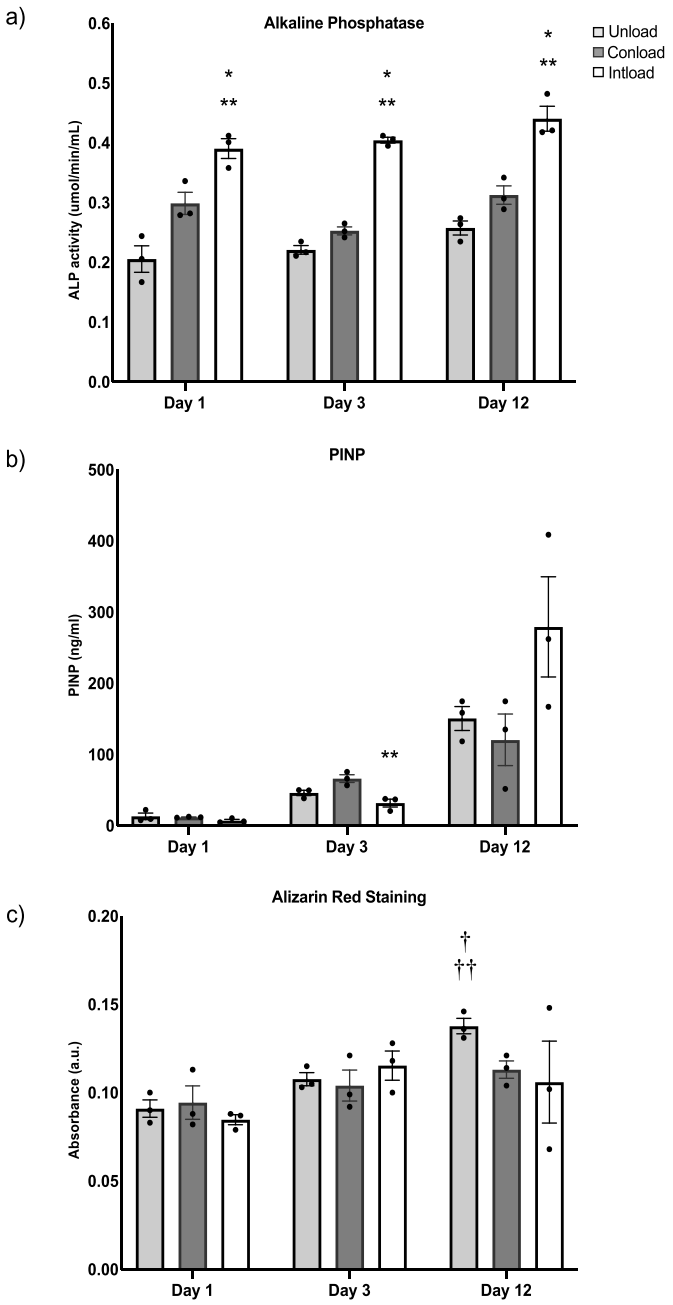

Fig. 3. a) ALP activity b) PINP activity and c) ARS absorbance across loading conditions. *p < .05 compared to unloaded. # <0.05 compared to continuous loading. Error bars represent standard error means.

3.3. Alizarin red staining

There was a significant difference in timepoint ($P = .006$) between groups in ARS. Post hoc analysis found a significant difference between days 1 and 12 ($p < .001$) and days 3 and 12 ($P = .007$) in the unloaded group. No significant differences were shown between loading conditions (Fig. 3c).

4. Discussion

Bone formation marker ALP was 30%–90% higher in the intermittent load condition compared to continuous load when loading magnitude was matched. Our data support the hypothesis that the osteogenic effects of implementing rest periods between loading cycles facilitate an increased response in bone formation (Burr et al., 2002; Robling et al., 2001) when observing ALP. It is speculated that the unloaded periods allow mechanosensitivity to be restored following the loading period and lead to heightened osteoblast activity.

This is the first study to apply the same loading parameters (magnitude, duration, frequency) across intermittent and continuous loading *in vitro,* and report the osteogenic potential of intermittent load on osteoblast activity. The osteogenic effect of intermittent loading shows the extent to which mechanosensitivity can be restored to enable the optimal stimulation of osteoblasts. The present study supports previous findings in rodents where the utilisation of 10s rest periods between low-magnitude loading cycles caused an increase in bone formation in comparison to continuous load (Srinivasan et al., 2003; LaMothe and Zernicke, 2004). One critical difference between previous studies and the present study is the difference in loading magnitude between conditions. For example, George et al. (2022) exposed rats to continuous (45 min/day at 70% maximal aerobic speed) and intermittent (42 min/day at 50–100% maximal aerobic speed) running for 8 weeks. Although similar protocols, the continuous and intermittent conditions are likely to have inflicted different cumulative loads, which may have been the cause for the difference in bone response rather than the addition of rest periods. Similarly, LaMothe and Zernicke (2004), did not match loading conditions, as the intermittent group were exposed to 10 fewer loading cycles compared to the continuous loading group. The present study subjected osteoblasts to the same load (5000 μS = 0.5% elongation), which is previously noted as a physiological strain that allows for optimal formation (Baudequin et al., 2019), as well as the same frequency (1 Hz) and duration (5 h). Intermittent loading was found to produce higher levels of ALP activity compared to the continuously loaded condition. As the loading magnitude was matched between conditions in the present study, it is therefore likely that the loading application contributes to the bone marker response. Mechanical loading protocols designed to induce osteogenic effects are an attractive means to combat osteoporosis. The present data suggests high-frequency loading used intermittently may augment osteogenesis by increasing osteoblast activity. In practical terms, this suggests the monotonous nature of activity such as running is suboptimal for bone accrual. It is therefore suggested that exercise programmes designed to improve bone health should incorporate rest periods.

The current study explored the effects of long-term rest periods compared to short-term rest periods *in vitro*. Previous research has shown longer rest periods (7 h) allow osteoblasts to restore their mechanosensitivity between loading bouts by increasing the expression of bone formation marker cyclooxygenase-2 (COX-2) present in early stage formation (Jaasma et al., 2007). Long-term rest periods of 30 min are also shown to enhance calcium response (Godin et al., 2007). Similarly, oscillatory flow with short-term rest insertion is shown to increase the frequency and size of calcium transients and upregulate intercellular calcium (Donahue et al., 2003). Short-term rest periods (5–15 s) incorporated within loading bouts are also shown to promote osteoblast activity by increasing osteopontin compared to continuous load (Batra et al., 2005). The present study supports previous evidence of stimulating osteoblast activity by showing increases in ALP during intermittent load suggesting longer resting periods might provide an osteogenic regime for osteoblast stimulation *in vitro*.

Bone formation marker PINP was lower in the intermittent loading condition compared to continuous loading on day three (Fig. 3b) however no differences were observed on day 12. This suggests osteoblasts may not respond positively to loading in the acute period. The acute variability in PINP response may be due to it being an indicator of matrix deposition and therefore unlikely that its activity levels will peak in the hours following an intervention (Dolan et al., 2022). This premise is supported by studies showing no difference in the PINP response to acute exercise 24 h (Evans et al., 2020; Dror et al., 2022; Kouvelioti et al., 2018) and 72 h (Scott et al., 2011) post-intervention. However, a local effect of loading on bone formation undetected by the marker

cannot be ignored, as findings from humans (Vainionpää et al., 2009) and animals (Zhang et al., 2011) imply loading may promote osteogenesis without detecting changes in PINP. Due to practical reasons, the *in vivo* assessment of PINP greater than 72 h post-intervention is not commonly conducted and therefore the present findings cannot be compared to human studies. The present findings suggest that loading does not have any effect on PINP concentration. Alizarin red staining (ARS) was also measured during the current study. ARS assay identifies calcium deposits that signify mineralisation. No differences were observed during either of the loading conditions yet, there was a significant difference over time in the unloaded condition. However, this method demonstrates moderate sensitivity meaning early differentiation or slight differences in mineralisation are difficult to detect (Serguienko et al., 2018). As the current study was conducted over 12 days it may be premature to identify significant mineralisation from the osteoblasts as it is proposed mineralisation is not detected until after 16 days in MC3T3 cells (Quarles et al., 1992). The differences in ARS observed in the unloaded condition may be a false positive result as ARS has been suggested to produce results in the presence of calcium-binding proteins and proteoglycans (Bonewald et al., 2003).

4.1. Limitations

The data of the present study were limited to acute bouts of loading of up to 12 days, therefore, the bone response following this period is not known. This study suggests bone formation markers offer insight into osteoblast activity *in vitro*, however, human studies need to be examined to confirm if a similar response to intermittent load occurs *in vivo*. Further research is warranted to investigate the effects of intermittent exercise on bone adaptation in humans.

4.2. Conclusions

In summary, the present study shows intermittent load increases bone formation marker ALP compared to a continuous load when loading magnitude, frequency and duration are matched. It can be hypothesised that the intermittent nature of the loading allowed osteoblasts to resensitise and restore their mechanosensitivity ensuring a heightened osteogenic response. The findings may be of interest to researchers and practitioners exploring exercise programmes for optimising bone accrual in human participants.

CRediT authorship contribution statement

Reece Scott: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ian Varley:** Writing – review & editing, Supervision, Investigation, Data curation, Conceptualization. **Craig Sale:** Writing – review & editing, Supervision, Conceptualization. **Janelle Tarum:** Writing – review & editing, Methodology, Formal analysis. **Ruth James:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Cleveland T. Barnett:** Writing – review & editing, Supervision, Conceptualization. **Lívia Santos:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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