EEG frequency-tagging captures the neural integration of bilateral periodic thermonociceptive stimulation

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Abstract

Sustained periodic stimuli are known to elicit a periodic neural response (i.e. steady-state evoked potential) in the EEG frequency spectrum. These responses can easily be traced at their frequency of stimulation and corresponding harmonics using a "frequency-tagging" approach. To date, sustained periodic thermonociceptive stimuli have only been used on one extremity (e.g. right volar forearm) at a time. Extending this paradigm to a bilateral application would enable its use to study the sensory integration of concomitant nociceptive stimuli and cognitive modulations during e.g. spatial attention tasks. This study demonstrates that bilaterally applied slow sustained periodic sinusoidal thermonociceptive stimuli using two different frequencies of stimulation (i.e. f_1, f_2, one on each forearm) can indeed be differentiated at the neural level by eliciting two distinct periodic responses at the respective frequency of stimulation and their harmonics. Additionally, we showed that there seems to be an interaction between the neural populations involved in the response to these stimuli, marked by neural activity at intermodulation frequencies ($n^* f_1 \pm m^* f_2$). So far, this non-linear integration of sensory information has already been observed following visual and auditory stimuli, but never following thermonociceptive stimuli.

Keywords: bilateral stimulation, steady-state evoked potential (SSEP), thermonociceptive, pain, neural integration, intermodulation, frequency-tagging

1. Introduction

Sustained periodic stimuli are known to elicit a periodic neural response at the frequency of stimulation and its harmonics (Regan, 1989). This "frequency-tagging" paradigm has been used for years in sensory domains such as auditory (e.g. (Geisler, 1960; Pantev et al., 1996)), vision (see Rossion et al. (2020) for a comprehensive review) or rhythm perception (Nozaradan et al., 2018; Nozaradan et al., 2011), but has also been extended to tactile (Moungou et al., 2016) and crucially, nociceptive stimuli (Colon et al., 2012a; Colon et al., 2012b; Mouraux et al., 2011).

Subsequent investigations demonstrated that an ultra-slow sustained periodic sinusoidal thermonociceptive stimulation applied at 0.2 Hz leads to improved signal-to-noise ratio (SNR) in the response to the nociceptive stimulation. Moreover, these slow steady-state evoked potentials (SSEPs) reflect predominantly the activation of slowly-adapting C-fibers (Colon et al., 2017). Being thus related to the "secondary", burning and lasting sensation of pain as well as being implicated in e.g. tissue inflammation (Campbell & Meyer, 1983), these SSEPs could potentially aid in the understanding of the role of C-fibers in pain perception while reducing the confound of stimulus salience, which is inherently present in most brief pain stimulations (Iannetti et al., 2008).

While this ultra-slow frequency-tagging paradigm has subsequently been used in a number of scalp EEG investigations (Leu et al., 2023; Leu et al., 2025; Leu et al., 2024; Mulders et al., 2020), which further deepened the understanding of these periodic brain responses, they have always been applied unilaterally to one extremity of the participant and at most concomitant with a stimulus of another modality (Colon et al., 2014). Yet, these applications do not tap into the full potential of this paradigm; given the high SNR generated by this type of slow sustained stimulus, it would be highly suited to tease out otherwise relatively small differences in neural processing and integration of nociceptive stimuli. In particular, the concomitant and bilateral application of these stimuli would also allow us to assess characteristics of cognitive processing induced by e.g. spatial attention tasks (as demonstrated in other sensory modalities: (Colon et al., 2015; Giabbiconi et al., 2007; Porcu et al., 2013; Saupe et al., 2009; Toffanin et al., 2009)).

On another note, concomitantly applied sensory periodic stimuli can elicit a phenomenon called intermodulation (*within* the same sensory modality) or crossmodulation (*between* sensory modalities), which is thought to reflect an interaction between the applied stimuli, leading to the non-linear neural integration of the two input signals emerging at "intermodulation frequencies" in the EEG frequency spectrum (i.e. $n^* f_1 \pm m^* f_2$) (Regan &

Regan, 1988a; Regan & Regan, 1988b; Yang et al., 2016). While audiovisual cross-modulation at these specific frequencies has previously been observed (Regan et al., 1995), it has since failed to be replicated by others using concomitant audiovisual (Giani et al., 2012), visual-tactile (Colon et al., 2015) or nociceptive-visual and nociceptive-tactile stimuli (Colon et al., 2014). Conversely, intermodulation by integration of different streams of visual input has been frequently achieved (reviewed in Chen et al. (2024); Gordon et al. (2019); Xu et al. (2022)). Using vibrotactile stimulation, intermodulation has also been found for concomitant stimulation on the same hand, but not when both hands were stimulated (Pang & Mueller, 2015). Yet, to the best of our knowledge, intermodulation has never been evidenced following concomitant sustained periodic nociceptive stimulation.

Thus, the main aim of this investigation was to demonstrate that the bilateral and concomitant application of ultra-slow sustained periodic sinusoidal thermonociceptive stimuli at two different frequencies (f_1, f_2) would elicit a periodic response in the EEG frequency spectrum for both applied stimulation frequencies. If successful, we aimed to further explore the possibility of intermodulation (i.e. non-linear integration of the concomitant stimuli), a response which would emerge at "intermodulation frequencies", a combination of harmonics of f_1 and f_2 (i.e. $n^* f_1 \pm m^* f_2$).

2. Methods

This experiment was pre-registered on Open Science Framework (OSF, https://doi.org/10.17605/OSF.IO/KN5QE) prior to the beginning of data collection.

2.1. Participants

20 healthy participants (between 18 and 35 years old) were recruited via an established website and were remunerated with 15€ for the duration of the experiment (approximately 1.5 h). Two participants had to be removed from the data due to technical problems with the EEG recordings and were subsequently also removed from the behavioral data analysis. The remaining participants (n = 18) were 24.2 ± 3.5 (mean ± std dev) years old (range: 20-35) and 8 participants were female. Only right-handed participants were included in the investigation.

Exclusion criteria included regular use of psychotropic medication, intake of pain killers such as paracetamol and nonsteroidal anti-inflammatory drugs (NSAIDs) within 12h before the experiment, as well as any severe neurological diseases, psychiatric disorders, or recent upper limb trauma. All participants gave written informed consent prior to the beginning of the

assessment and were informed that they could withdraw from the experiment at any given time. All procedures were performed in accordance with the relevant guidelines and regulations. The local Research Ethics Committee approved all experimental procedures (Commission d'Ethique Hospitalo-Facultaire Saint-Luc UCLouvain, B403201316436).

The software G*Power was used to conduct a power analysis. We aimed to reach a power of 0.8 using a standard 0.05 alpha error probability. Based on data of Colon et al. (2017), we can expect a relatively large effect size (d=1.15) for the detection of a periodic response (tested against zero, one-sided) at the frequency of stimulation, resulting in a suggestion of 10 participants. To account for a potentially smaller effect size given the concomitant bilateral stimulation (as opposed to the commonly used unilateral 0.2 Hz stimulation), and to match investigations in other domains which observed responses at the intermodulation frequencies (Boremanse et al., 2013; Giani et al., 2012), we increased the sample size to 20 participants.

2.2. Thermonociceptive stimulation

Two thermal cutaneous stimulators (TCS II, QST.Lab, Strasbourg, France) - each equipped with the T03 probe - were used to deliver sustained periodic thermonociceptive stimuli on the volar forearms of the participants. The probes were set with 15 micro-Peltier elements (each ~7.7 mm2, arranged in a 30-mm diameter circle), whose temperature can vary at rates of up to 300 °C/sec. The temperature of stimulation varied between 35 °C and 50 °C in a sustained periodic sinusoidal manner (Figure 1). Two frequencies of stimulation were applied for 60 sec per trial, one on each arm of the participant: Frequency 1 (f_1) contained cycles of 4 sec (i.e. at 0.25 Hz), therefore accumulating 15 cycles within one trial. Frequency 2 (f_2) was delivered at 0.333 Hz, with each cycle lasting 3 sec which was repeated 20 times within a trial (Figure 1). The forearm on which f_1 and f_2 were applied was counterbalanced, so that the left and right arm received the same number of stimuli with both stimulation frequencies across the experiment. Inter-stimulus-intervals were variable and self-paced by the experimenter to allow participants to provide ratings of intensity and painfulness. The thermode was displaced after each trial to avoid habituation or sensitization. Stimulation frequencies were determined based on previous studies demonstrating that ultra-slow sustained periodic thermonociceptive stimuli have a superior SNR in frequency-tagging paradigms compared to faster paced thermonociceptive stimuli (Colon et al., 2017). Additionally, the frequencies were chosen so that their harmonics would not overlap in the frequency spectrum. Lastly, we aimed to have stimulation frequencies that are not too different from each other, to make it more difficult for the participants to differentiate the applied stimulation frequencies and to avoid delivering significantly more

stimulation cycles on one arm than on the other during the same trial (i.e. avoiding confounds due to e.g. temporal summation).

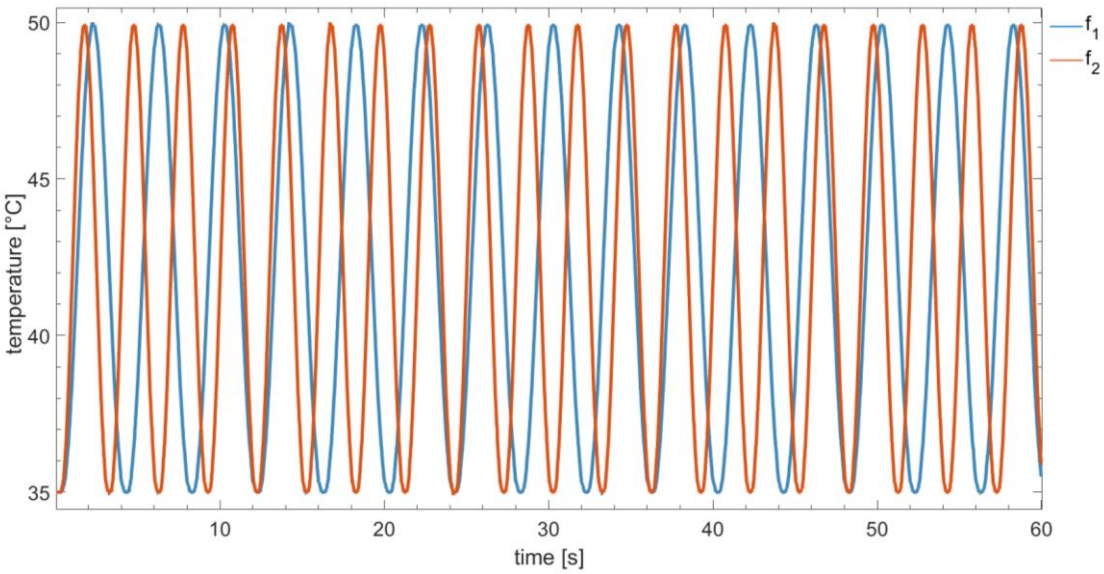

Figure 1. *Illustration of the bilateral and concomitant stimulation. Sustained periodic sinusoidal thermonociceptive stimuli were delivered at the frequencies f_1 (0.25 Hz) and f_2 (0.333 Hz), delivered using a thermal cutaneous stimulator (TCS II, QST.Lab, Strassbourg, France).*

2.3. Experimental design

Participants were seated comfortably in a chair in front of a table, on which both their forearms were resting with the volar surface upwards. They were instructed to move as little as possible and keep their gaze constant to avoid interference with the EEG signal acquisition during the thermonociceptive stimulation. Additionally, they were encouraged to focus their attention on the bilaterally applied stimulation during the trials. In total, 30 trials of the bilateral thermonociceptive stimulation were delivered, split into 6 blocks of 5 stimulation trials. The arm on which f_1 and f_2 were applied was randomized and counterbalanced, so that each arm received an equal amount of stimulation using f_1 and f_2.

2.4. EEG recordings

An elastic electrode-cap with 64 active, pre-amplified Ag-AgCl electrodes (BioSemi, Netherlands) arranged in accordance with the international 10-10 system was used to record EEG with a sampling rate of 1024 Hz. To maintain a clear signal, the direct-current offset was limited to 30 mV. All electrodes were re-referenced offline to the average electrode activity. The BioSemi ActiView software stored the recorded signal for subsequent offline analyses.

2.5. EEG analysis

The EEG recordings were preprocessed using the Letswave7 (www.letswave.org) toolbox and analyzed using custom scripts in MATLAB (2022a The MathWorks).

2.5.1. Preprocessing

We employed a frequency-tagging analysis approach (Regan, 1966; Regan, 1989) to analyze the periodic response induced by the slow sustained periodic stimuli, which allows for an objective differentiation between oscillatory activity related to the applied stimuli and other unrelated ongoing activity (Colon et al., 2012b). The frequency-tagging method is based on the notion that a periodic stimulus elicits a periodic activity which can be identified as periodic responses at the frequency of stimulation in the recorded EEG signals (Colon et al., 2012a; Mouraux et al., 2011). This approach has been frequently used in our lab, leading to a standardized analysis approach (Colon et al., 2017; Leu et al., 2023; Leu et al., 2025; Leu et al., 2024; Mulders et al., 2020).

The obtained EEG signal was first filtered using a fourth-order Butterworth band-pass filter between 0.05 and 100 Hz, then a Notch filter with a 2 Hz width was applied at 50 and 100 Hz. The signal was segmented into epochs of the length of stimulation (60s), relative to the onset of the stimuli. Due to their placement on the EEG cap, electrodes Iz, P9 and P10 are often very noisy and were therefore removed from the data set to improve signal quality. To remove potential muscular artifacts (i.e., from eye movements), an Independent Component Analysis (Fast ICA algorithm) (Hyvarinen & Oja, 2000) was applied, and any trial containing amplitudes larger than ± 500 µV was removed. On average, ~ 3 ± 2 independent components supposed to reflect noise signals were removed per participant. Less than 1% of trials were removed overall due to large artifacts (amplitude > 500 µV) that could not be removed using the ICA. Noisy channels were interpolated using their 3 neighboring electrodes. The remaining signal was re-referenced to the average of the electrode set and epochs were concatenated to a length of 120 sec, to improve the signal resolution in the frequency domain (frequency resolution = 1/epoch duration, i.e., 1/120 sec = 0.00833 Hz). The epochs were then averaged across participants. To analyze the signal in the frequency domain, a discrete Fourier transform (FFT) (Frigo & Johnson, 1998) was used. Finally, residual noise was partially removed by subtracting the average amplitude of the signal measured at 2–8 neighboring frequencies (thereby excluding the bins immediately adjacent to the bin of interest), at each electrode and at each frequency bin (Mouraux et al., 2011).

2.5.2. *Aggregation of harmonics*

The neural response to such periodic stimulation is known to be distributed across harmonics in the frequency spectrum, and these harmonics should be aggregated (i.e., summed up) for an adequate reflection of the neural response to the stimulation (Retter et al., 2021). While there are many different approaches on how to define the ideal number of harmonics to sum, we chose to find the harmonic at which the aggregated signal has the highest signal-to-noise ratio (SNR) in the frequency spectrum, using baseline-corrected group- and channel-averaged data for f_1 and f_2. The SNR was calculated by dividing the signal at a given frequency bin *x* by the mean amount of noise at the 8 surrounding frequency bins (*x/ baseline noise*, excluding immediately adjacent bins). This method differs slightly from previous investigations from our lab, where the harmonics were either not taken into account (Colon et al., 2017; Mulders et al., 2020) or all harmonics in the frequency spectrum were aggregated, regardless of their contribution to the SNR (Leu et al., 2023; Leu et al., 2024). We chose a compromise, minimizing the possibility of disregarding relevant parts of the brain's response to the applied stimuli while reducing the amount of potential noise that would be aggregated into the signal.

To aggregate the harmonics, a custom MATLAB script was used to sum up the amplitude at each harmonic of each frequency of stimulation consecutively and assess the new SNR at each step (evaluated against 8 frequency bins on each side). Based on this evaluation, the highest SNR was identified at 9.25 Hz for f_1 and 7.333 Hz for f_2. Similar to other thermonociceptive SSEP investigations, the first few harmonics carried most of the periodic neural response (Colon et al., 2017; Leu et al., 2023; Leu et al., 2025; Leu et al., 2024; Mulders et al., 2020).

A multi-sensor cluster-based Wilcoxon signed-rank test (clusters built using up to 4 electrode neighbors, 1000 permutations, $p<0.05$) was used to objectively assess the electrodes with the largest responses (i.e. amplitude) at the aggregated harmonics for f_1 and f_2 separately.

2.5.3. *Assessment of intermodulation responses*

The baseline-corrected signal was grand averaged across participants, and z-scores were calculated at each frequency bin by calculating the difference between the amplitude of the frequency bin and the averaged amplitude of the 8 frequency bins on each side (16 in total), excluding the immediately adjacent bins, and divided by the standard deviation of these 8 bins (following the formula: $x - \textit{baseline noise} / \textit{std dev}_{\textit{baseline noise}}$).

Significant z-scores at intermodulation frequencies (i.e. $n^* f_1 \pm m^* f_2$, where *n* and *m* are integers) were identified in the group- averaged EEG spectrum up to 50 Hz at each channel *($p<0.001$).* For each channel and participant, the baseline subtracted amplitudes at the

significant intermodulation frequencies of that channel were extracted and (after excluding direct harmonics of f_1 and f_2) aggregated to form the "intermodulation response" at that channel. Thus, for each channel, a different number of intermodulation harmonics was aggregated to create an accurate reflection of the neural response to the bilateral stimuli across the scalp.

2.5.4. Assessment of number of trials necessary for a good SNR

A larger number of trials were used in this experiment than in previous frequency-tagging investigations from our lab. To explore how many trials would suffice for a good SNR at the frequency of stimulation and harmonics of f_1 and f_2 (excluding the intermodulation response), we ran the analysis for either the first 6, 12, 18 and 24 trials of each participant and assessed the SNR of the baseline-subtracted group- and channel-averaged signal at the subsequently aggregated harmonics in the frequency spectrum

2.6. Statistical analysis

Statistical analyses were carried out using R Statistical Software (Version 4.3.1, R Core Team 2023) and MATLAB (2020b The MathWorks). The significance level was set at $p \leq 0.001$ for the identification of significant intermodulation responses, consequently z-scores ≥ 3.1 were considered as significant (one-sided, signal > noise). For other statistical tests the significance level was set to $p \leq 0.05$.

Linear mixed models were used to assess differences in intensity and pain ratings across the stimulation blocks as well as to evaluate whether amplitudes elicited by stimulation with f_1 and f_2 differ from each other at different harmonics. Post-hoc pairwise t-tests were applied to inform about the direction of any significant effects.

3. Results

This study investigated whether bilaterally and concomitantly applied slow sustained thermonociceptive stimuli at frequencies f_1 and f_2 lead to a periodic neural response at both frequencies of stimulation, as well as potentially leading to neural integration of the signals at intermodulation frequencies. Apart from 64-channel EEG, ratings of pain and intensity perception were recorded after each stimulus.

3.1. Behavioral response

On average, the stimuli were perceived as painful in 35 % of the trials (Figure 2a). 5 participants never perceived the stimulation as painful, while for most other participants the number of trials

that were perceived as painful varied between the stimulation blocks. A slight increase in the number of trials which were perceived as painful (compared to the pain evaluation in block 1) was noted in blocks 4 to 6, with the largest increase in block 5 ($t_{B4}(89) = -2.467$, $p=0.016$; $t(89)_{B5} = -2.782$, $p = 0.007$; $t_{B6}(89) = -1.996$, $p = 0.049$). All participants indicated that they perceived the stimuli at a similar intensity on both arms. Overall, the stimuli were evaluated at an intensity of 5.33 ± 2.28 (mean ± std dev). To be able to compare ratings between participants, the ratings of each participant relative to their ratings in the first stimulation block were calculated (see Figure 2b). A significant effect of stimulation block was found in the LMM ($F(5, 569.01) = 2.525$, $p = 0.0283$, $\eta_p^2 = 0.02$). Post-hoc pairwise comparisons revealed a significant increase in relative ratings in blocks 4 to 6 compared to block 1 ($t_{B4}(89) = -2.632$, $p=0.01$; $t_{B5}(89) = -3.135$, $p = 0.002$; $t_{B6}(89) = -3.698$, $p < 0.001$).

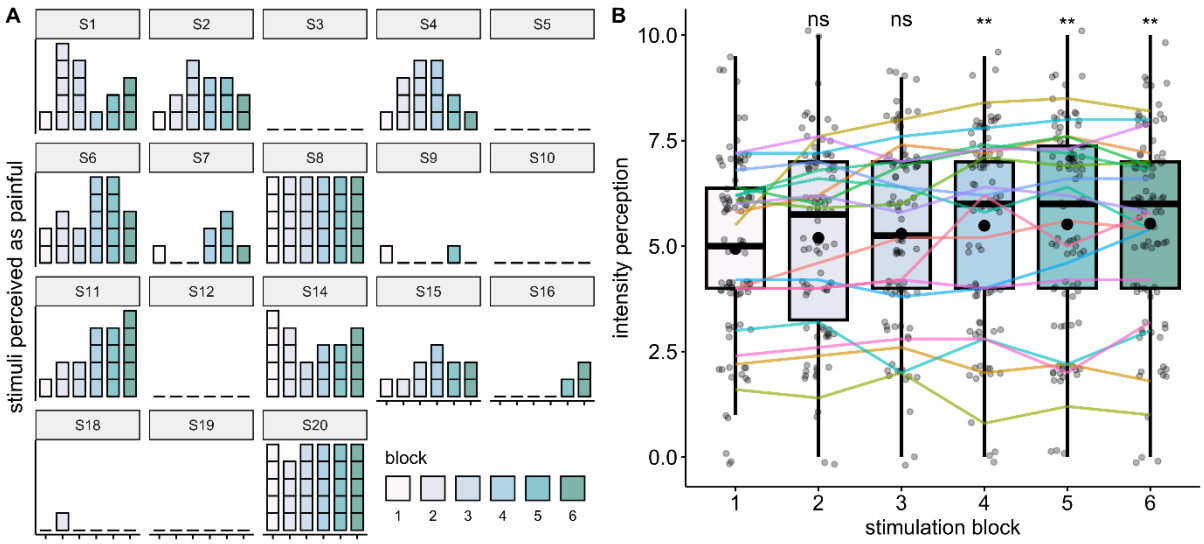

Figure 2. *Ratings of perceived painfulness and intensity of the heat pain stimuli.* ***a)*** *Illustration of the number of trials that were perceived as painful for each participant and each stimulation block.* ***b)*** *Ratings of perceived stimulation intensity in each block. Horizontal lines indicate the evolution of each participants' average rating per block, while the mean for each block is indicated using round black dots. Individual data points are jittered around the boxplots. Results of pairwise t-tests comparing each block to block one are indicated: ^{ns} p>0.05, ** p<0.01, *** p<0.001.*

3.2. EEG response

Stimuli applied with frequency f_1 and f_2 elicited clear periodic responses at their respective frequency of stimulation and their harmonics (Figure 3). The first few harmonics were the largest for both stimulation frequencies, but they were distributed slightly differently across the frequency spectrum, leading to a different number of harmonics that were aggregated to achieve the highest SNR for the response to each stimulation intensity.

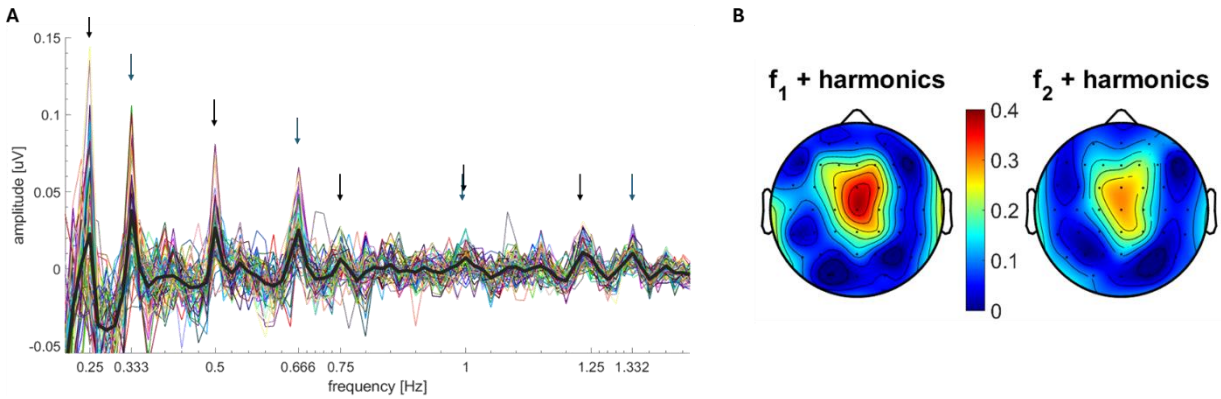

Figure 3. *Neural response recorded following concomitant and bilaterally applied sustained periodic thermonociceptive stimulation on the volar forearms.* ***A)*** *Steady-state evoked potentials elicited by stimuli at frequency f_1 (.25) and f_2 (.333) in the EEG frequency spectrum. Group averaged data are indicated for each channel (colored) and the pooled channels (black).* ***B)*** *Topographical plots represent the response at the aggregated harmonics for f_1 and f_2, based on the number of aggregated harmonics with the highest SNR in the frequency spectrum estimated from group- and channel-averaged data for f_1 and f_2.*

The aggregated harmonics demonstrated activity mainly distributed around the scalp vertex (f_1: W(Cz) = 3.40; f_2: W(Cz) = 3.57) at both frequencies of stimulation (Figure 3B) as well as at temporal channels in f_1 (f_1: W(TP7) = 3.70; W(T8) = 3.48). The aggregated amplitudes at the scalp vertex elicited by stimulation with f_1 were slightly larger from the ones elicited by f_2 ($F(1,17) = 6.336$, $p = 0.022$, $\eta_p^2=0.27$). Interestingly, if the same number of harmonics are taken into account fpr both stimulation frequencies (largest common denominator: 22 harmonics), this difference is not present anymore ($F(1,773) = 1.941$, $p = 0.164$, $\eta_p^2=0.002$).

As we used a rather large number of trials in this experiment, we also explored how many trials would suffice to achieve a good SNR, having in mind that future studies will likely employ different experimental conditions while using bilateral stimuli. This sub-analysis on the first 6, 12, 18 and 24 trials of the experiment revealed that applying a minimum of 12 trials for a comparatively good SNR for stimuli using f_1. Interestingly, stimulation frequency f_2 seems to have overall a higher absolute SNR if more than 18 trials are included. The SNR of 12 trials is similar to f_1 with the same number of trials (Figure 4).

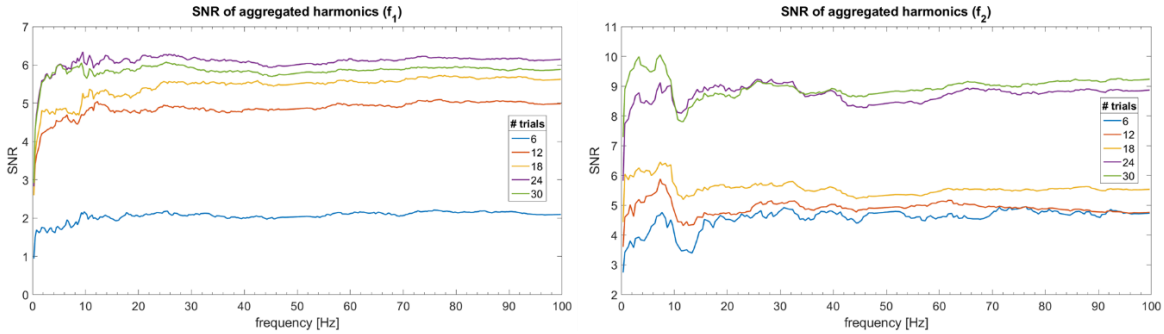

Figure 4: *SNR development across the frequency spectrum for different numbers of trials. For each frequency of stimulation, the number of trials averaged before the frequency transformation was gradually decreased to assess its impact on the SNR across the frequency spectrum. The aim was to find the lowest number of trials still achieving a comparatively high SNR.*

Significant intermodulation frequencies were identified based on their z-scores, separately for each channel. The amplitudes at these frequencies were aggregated to quantify the intermodulation response across the scalp. The largest intermodulation responses were found at parietal-central channels (Figure 5A). Most participants exhibited an intermodulation response (signal > noise) at almost all channels, with multiple harmonics being present (see example of channel Pz in Figure 5B and 5C)

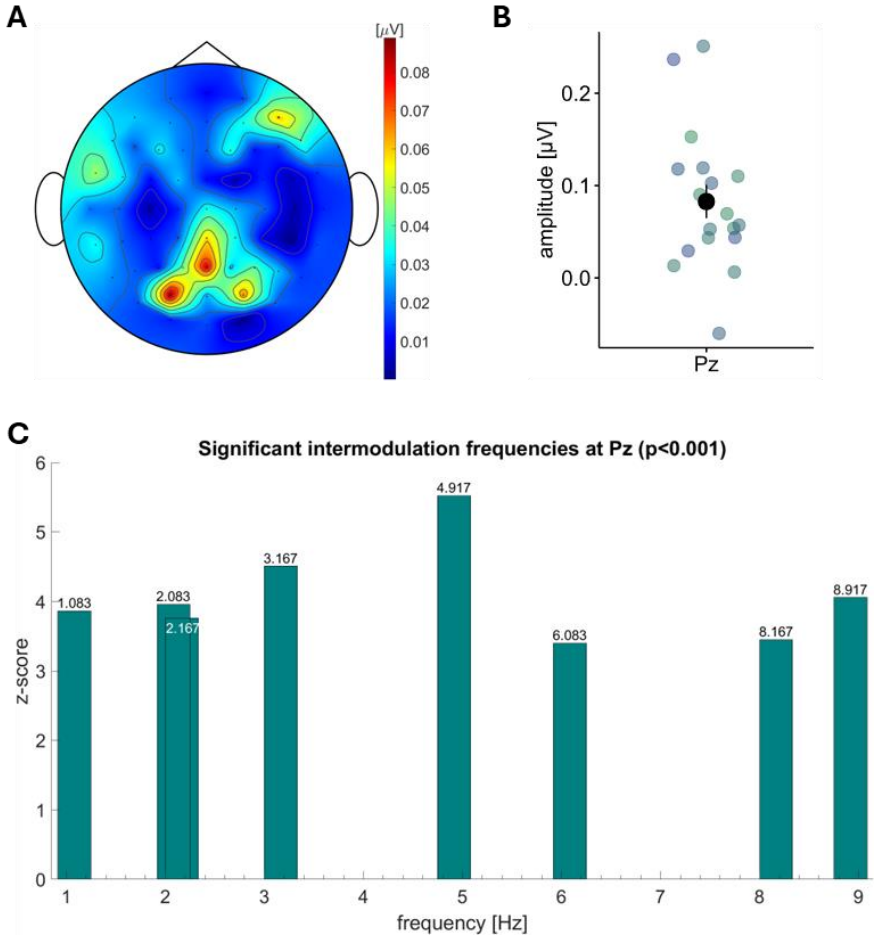

Figure 5*. Neural activity at intermodulation frequencies.* ***A****) Topographic illustration of the group-level amplitude of the aggregated significant intermodulation frequencies for each channel.* ***B)*** *Individual participant aggregated baseline-subtracted amplitudes at channel Pz.* ***C)*** *Group-level z-scored responses at electrode Pz exceeding the threshold of z ≥ 3.1 (p < 0.001) at intermodulation frequencies up to 10 Hz. The specific frequencies of the significant z-scores are indicated in the plot.*

4. Discussion

4.1. Bilateral frequency-tagging of sustained periodic thermonociceptive stimuli

This proof-of-concept study aimed to demonstrate that bilateral and concomitant application of ultra-slow sustained periodic sinusoidal thermonociceptive stimulation at frequencies f_1 and f_2 elicits periodic neural responses (i.e. SSEPs) at each frequency of stimulation (and its harmonics), which can objectively identified using a frequency-tagging approach. Additionally, we investigated whether the simultaneous activation of potentially similar neuronal populations by the bilateral stimulation leads to a non-linear integration of the nociceptive input, which would be present at intermodulation frequencies (i.e. $n^* f_1 \pm m^* f_2$).

Both stimuli elicited clear SSEPs in the frequency spectrum at the frequency of stimulation and their harmonics. The largest periodic response to these concomitantly applied ultra-slow stimuli was found at the **scalp vertex** for both stimuli, consistent with other thermonociceptive SSEP investigations (Colon et al., 2017; Leu et al., 2023; Leu et al., 2025; Leu et al., 2024; Mulders et al., 2020), and likely reflecting activity of the ACC as well as left and right operculo-insular cortices (Mouraux et al., 2011; Owen et al., 2010; Peyron et al., 2000).

The **magnitude of the aggregated SSEPs** was similar between stimuli at f_1 and f_2 and similar to other thermonociceptive SSEP investigations, the first few harmonics carried most of the periodic neural response (Colon et al., 2017; Leu et al., 2023; Leu et al., 2025; Leu et al., 2024; Mulders et al., 2020). Comparing the obtained responses to those previous investigations, it seems that the amplitude of the SSEPs evoked by bilateral stimuli are somewhat lower than their unilateral counterparts in comparable conditions (Colon et al., 2017; Leu et al., 2023; Mulders et al., 2020). Similar results have been found after the bilateral and simultaneous application of brief thermonociceptive laser stimuli; although more nociceptive input reached the cortex, their neural representation (i.e. N2-P2 amplitude) was decreased regardless of the direction of attention (here: left or right hand) (Northon et al., 2019). Contrasting results were found by the same group, where they observed an increase in N2 and P2 magnitude during bilateral laser stimuli, an effect that was stronger when the hand were positioned closer to the participant's body (Northon et al., 2022). Yet, even within this investigation, about 30% of participants actually exhibited a decrease of their N2-P2 amplitudes, showcasing the large heterogeneity in the neural processing of bilateral thermonociceptive stimuli. While our frequency-tagged responses are not identical to an event-related potential , the phase-locked

response which was considered in the present investigations is thought to largely reflect similar properties as an ERP elicited by the same stimulation (Courtin & Mouraux, 2024 Preprint).

Since a relatively large number of trials were used in this experiment, we wanted to explore how reducing this number would affect the SNR at the frequencies of stimulation and their harmonics. This is particularly important as future investigations are likely to employ multiple conditions, and it will not be feasible to accumulate this many trials for each condition without negatively impacting the subject (i.e. discomfort) or the experiment (i.e. duration). The assessment of the SNR using less than 30 trials showed that a minimum of 12 trials should be applied for a good SNR at both frequencies of stimulation, corresponding to similar experiments that apply the sustained periodic thermonociceptive stimuli to only one arm.

Overall, the magnitude of the **perception of stimulus intensity** was comparable to other investigations using a similar rating scale and (unilateral) sustained periodic heat stimuli (Colon et al., 2017; Leu et al., 2023; Leu et al., 2024). Our results also falls in line with other investigations applying bilateral brief heat stimulation (simultaneous rather than concomitant), which found no change in pain perception between uni- and bilateral stimulation of the hand using a focal rating (Northon et al., 2021; Northon et al., 2019). Yet, while not affecting pain-related EEG measures, spatial attention seems to modulate the perception of bilaterally applied stimuli, as ratings of “global” perception (i.e. both hands rated together) led to higher evaluations than a “focal” (only one hand) ratings (Northon et al., 2022). Conversely, the perception of pain-related SSEPs applied through high-frequency transcutaneous electrical stimuli did not exhibit a change depending on the direction of the attention, demonstrating the difficulty to shift attention if stimuli are applied at the same time (Blöchl et al., 2015). Future investigations should probe whether this holds up also when stimuli are delivered in a sustained and concomitant manner, as in the present paradigm.

The intensity perception of the stimuli seemed to increase slightly over the course of stimulation blocks, with significantly higher ratings compared to the first block in blocks 4 to 6. This is likely related to the phenomenon of **peripheral sensitization**. While the location of the thermode was shifted after each stimulus, given the total number of 30 stimuli, it cannot be completely avoided to stimulate similar areas multiple times, which potentially led to spatial summation (Nielsen and Arendt-Nielsen, 1997). Other investigations using a similar stimulation paradigm found a trend for habituation of continuous ratings within one trial (i.e. during the 75s of sustained stimulation), not sensitization (Mulders et al., 2020). Sensitization might have been less present in other thermonociceptive SSEP investigations due to changes in temperature between different conditions (i.e. not all trials of an experiment were applied using the same

stimulation temperatures) as well as shorter overall duration of the stimuli across the experiment (i.e. due to shorter or less trials). Additionally, these investigations never directly investigated the change in intensity perception over the course of the experiment, which makes comparisons difficult.

Curiously, the number of trials **perceived as painful** is about half of the percentage of trials reported as painful in those investigations (Leu et al., 2023; Leu et al., 2024), despite similar levels of intensity perception. The repetitive and predictive nature of the applied stimuli might have contributed to a decrease in the level of "perceived bodily threat" of these stimuli. In the previously mentioned thermonociceptive SSEP investigations, variations in conditions were always present, therefore leading to a more variations in the perception and reducing the predictability of the stimuli.

A limitation of this experiment is that the chosen frequencies of stimulation, while never reaching the stimulation peak at the same exact time, are rather complex to assess in the frequency spectrum given the frequency of f_2. Errors due to rounding, and the limited resolution in the frequency domain (meaning that harmonics "fall" into frequency bins left or right of where they are supposed to be) complicates the analysis and evaluation of the response. An improvement for future investigations would be to use a similarly slow frequency of stimulation, but with a simpler number, e.g. 0.4 Hz.

4.2. Intermodulation

With the successful implementation of the bilateral frequency-tagging using thermonociceptive stimuli, we set out to identify significant responses at intermodulation frequencies, which would illustrate the non-linear neural integration of the concomitantly applied stimuli. Since the neural activity elicited by these stimuli was most prominent at the scalp vertex, we expected that their activity might "overlap" and create an interaction, leading to the neural integration of the stimuli. This hypothesis was also supported by the perception of the participants, which – while not necessarily being perceived "as one" – also did not differ between the stimuli. Using a standard z-score threshold we found a large number of significant z-scored frequency bins across the frequency spectrum. Thus, to be more selective and forego issues of multiple comparison, we increased the significance threshold to a more conservative $p < 0.001$. Still, most channels showed multiple significant intermodulation harmonics, predominantly at higher harmonic combinations (i.e. larger than 1Hz).

The main difference to previous attempts at investigating the non-linear integration of nociceptive stimuli might lie in the improvement in SNR (Colon et al., 2017). In the first attempts

to elicit SSEPs through nociceptive stimulation, relatively fast (> 3 Hz) thermonociceptive (Colon et al., 2014; Mouraux et al., 2011) and electrical (Colon et al., 2012b) stimuli were used, predominantly activating Aδ-fibers with maximal responses over fronto-central regions. Comparing these responses to concurrently applied tactile and visual stimulation showed that each modality elicited activity in a distinct subgroup of neuronal populations (Colon et al., 2012a), but that selectively attending either thermonociceptive or tactile stimulation did not alter the magnitude of the SSEP responses (Colon et al., 2014). One caveat of these studies was the relatively low SNR in the response to the high-frequency sustained periodic thermonociceptive stimuli. Given the improved SNR using ultra-slow stimuli (Colon et al., 2017), it could be interesting to revisit previous attempts at investigating potential cross-modal interactions between activation of the nociceptive system and other sensory stimuli and thereby improve our understanding of how a salient and threatening stimulus modulates and integrates with other sensory information.

The phenomenon of intermodulation following concomitant and periodic sensory input is not new. In the visual domain, the usefulness of neural integration of two different stimuli has elegantly been demonstrated by periodically presenting half-faces at different frequencies, during which the brain tries to integrate the information to a complete face (Boremanse et al., 2013, 2014). Comparatively, it is less clear what the responses recorded at intermodulation frequencies reflect in the context of bilateral thermonociceptive stimulation. Usually, intermodulation frequencies can be found only few harmonic combinations away from their "base" frequency (e.g. $1*f_1+1*f_2$). This was not the case in this investigation, where intermodulation frequencies were identified predominantly at higher harmonic combinations, which raises the question of what processes these responses reflect. The fact that no intermodulation responses were found at early harmonic combinations in the z-scored signal shows that these responses were relatively small. These responses potentially reflect interhemispheric coherence as the brain was integrating the sensory input ascending from each arm.

Additionally, the fact that the stimulation was never truly simultaneous (i.e. the applied heat cycles never peaked exactly at the same time, though close to each other) could have also hampered a potential "cleaner" neural integration.

While this investigation will not be able to answer this question conclusively by itself, we hope that these results can lay the foundation for future investigations probing how the human brain integrates concomitant activation of the nociceptive system or in combination with other sensory modalities.

5. Conclusion

This investigation successfully demonstrated that sustained periodic thermonociceptive stimuli applied concomitantly to both forearms at a frequency f_1 and f_2 elicit a periodic neural response at both frequencies of stimulation, easily identifiable using frequency-tagging. This opens the door to extending the frequency-tagging paradigm to a bilateral application of (thermo-)nociceptive stimuli, a promising paradigm to better understand the neural representation and integration of concomitant nociceptive stimuli.

We further demonstrated the presence of periodic responses at intermodulation frequencies, which evidences the non-linear integration of the concomitantly applied thermonociceptive stimulation.

Thus, using slow sustained periodic thermonociceptive stimulation could be beneficial not only to better understand nociceptive processing by itself, but also its cognitive modulation and multisensory integration in relation to other sensory stimuli.

Acknowledgments

We wish to thank Francesca Barbero and the NOCIONS research group for their helpful feedback and suggestions.

Funding

CL and GH were each supported by a FRIA doctoral grant by the Belgian Fund for Scientific Research (F.R.S.–FNRS). VL was also supported by the F.R.S.-FNRS.

Conflict of interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization : **CL, GH, VL;** Methodology : **CL, GH;** Investigation : **CL, GH;** Validation : **CL;** Formal analysis : **CL;** Data Curation : **CL;** Visualization: **CL;** Supervision : **VL, GL;** Funding acquisition : **VL;** Writing - Original Draft: **CL;** Writing - Review & Editing: **CL, GH, GL, VL**

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