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*Published in:*  
European Journal of Pain

*DOI (link to publication from Publisher):*  
[10.1002/ejp.1279](https://doi.org/10.1002/ejp.1279)

*Publication date:*  
2018

*Document Version*  
Accepted author manuscript, peer reviewed version

[Link to publication from Aalborg University](#)

*Citation for published version (APA):*  
Høgh, M., Petersen, K. K., & Graven-Nielsen, T. (2018). Effects of repeated conditioning pain modulation in healthy volunteers. *European Journal of Pain*, 22(10), 1833-1843. <https://doi.org/10.1002/ejp.1279>

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**Article Type: Original Manuscript**

## **EFFECTS OF REPEATED CONDITIONING PAIN MODULATION IN HEALTHY VOLUNTEERS**

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**Original publication for Eur J Pain**

**Keywords:** conditioned pain modulation, pain inhibition, cuff algometry, diffuse noxious inhibitory controls, endogenous pain inhibition.

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**Funding sources:** Center for Neuroplasticity and Pain (CNAP) is supported by the Danish National Research Foundation (DNRF121).

**Conflicts of interest:** Nocitech is partly owned by the Aalborg University.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/ejp.1279

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**Significance:** Conditioning pain modulation (CPM) can be assessed in 5-minute intervals by cuff algometry with a fixed conditioning stimulus. Without applying conditioning stimuli the pain sensitivity of test-stimuli habituated. As a consequence, it can be speculated that the conditioning stimulus may negate the temporal habituation effects during repeated sessions, whereas this may not be the case for unconditioned stimuli. Applying both conditioned and unconditioned repeated test-stimuli may be a way to assess different parts of the pain modulatory system, and a model for measuring a netCPM-effect, which could indicate a balance between habituation and sensitisation, is proposed.

## **ABSTRACT**

**Background:** Conditioned pain modulation (CPM) may be impaired in chronic pain patients compared with healthy subjects. The CPM-effect is the difference between pain sensitivity assessments (test-stimuli) with and without a painful conditioning stimulus. CPM has been extensively explored but effects of repeated CPM-effects and differences between repeated CPM assessments and comparable control conditions are less studied.

**Methods:** In 20 healthy men, four 5-min bouts with a test-stimulus in the beginning and midway were applied by cuff-algometry to the dominant leg. The 2<sup>nd</sup> test-stimulus in each bout was conditioned in parallel by a painful cuff-pressure on the contralateral leg. A control-session was performed without conditioning. The conditioning-intensity was 70% of the pressure-pain tolerance threshold (PTT) assessed at baseline. Pain detection threshold (PDT) was extracted from test-stimuli. CPM/Control-effects were calculated as second minus first test-stimulus, and netCPM-effects were calculated as the difference between CPM-effects and Control-effects.

**Results:** PDT increased in all four bouts ( $p < 0.02$ ) compared to the unconditioned test-stimulus and compared to the 2<sup>nd</sup> test-stimulus in bout1, bout3, and bout4 of the Control-session ( $p < 0.04$ ). In the Control-session, the 1<sup>st</sup> test-stimulus PDT increased from bout1 to bout2, bout3, and bout4 ( $p < 0.03$ ). The netCPM-effect increased progressively over the four bouts ( $p = 0.03$ ).

**Conclusion:** CPM-effects were maintained over four consecutive bouts and in the Control-session repeated pain thresholds assessments habituated more than in the CPM-session leading to an increase in netCPM-effect over the four bouts.

## INTRODUCTION

Pain is a dynamic phenomenon under influence of many factors including descending control mechanisms, which can be assessed by conditioned pain modulation (CPM) protocols in humans (Yarnitsky et al., 2015a). The CPM-effect is the degree of change in pain sensitivity assessed by a test-stimulus when a painful conditioning-stimulus is applied before or in parallel with the test-stimulus (Yarnitsky et al., 2015a). The conditioning stimulus should be applied heterotopically to the test-stimulus, indicating that CPM depends on central mechanisms (Klyne et al., 2015; Le Bars et al., 1992).

Intersession reliability of CPM-effects is good with interclass correlation coefficients (ICC) from 0.6-0.75, although the reliability depends on stimulation parameters, modality and protocols (Granovsky et al., 2016; Imai et al., 2016; Kennedy et al., 2016). Computerized pressure cuff-algometry has excellent reliability over days (ICC > 0.8) (Graven-Nielsen et al., 2015), both for unconditioned and conditioned test-stimuli (Graven-Nielsen et al., 2017; Imai et al., 2016). The cuff algometry CPM effect has also been reported with good reliability (Graven-Nielsen et al., 2017).

Impaired CPM-effect has been linked to local painful conditions including knee pain (Arendt-Nielsen et al., 2010; Rathleff et al., 2016) and widespread pain such as fibromyalgia (Gerhardt et al., 2017; Julien et al., 2005; Potvin and Marchand, 2016). Yet, some studies compare the conditioned test-stimulus to a single, unconditioned test-stimulus (Granovsky et al., 2016; 2017; Imai et al., 2016; Vaegter et al., 2017b) while others use an average of two or more test-stimuli to compare with a single conditioned test-stimulus (Rathleff et al., 2016; Skovbjerg et al., 2017; Stolzman and Hoeger Bement, 2016; Stolzman and Bement, 2016; Vaegter et al., 2017a). Interestingly, a study showed that migraineurs lose the pain-inhibitory effect of CPM when CPM-paradigms are repeated in three bouts with 40 seconds intervals, suggesting that insufficiencies in the descending system may not be detected with single-bout CPM assessment (Nahman-Averbuch et al., 2013). However, the study did not test repeated unconditioned test-stimuli and therefore leaves the question if repeated bouts of conditioned and unconditioned test-stimuli behave differently and possibly reflect different aspects of the descending modulation of pain.

Repeated, unconditioned, painful test-stimuli in healthy subjects was found to habituate with less than 30 seconds between stimuli (Yarnitsky et al., 2010) and a constant cuff pressure for 10 minutes led to pain adaptation in 72% of healthy subjects (Pud et al., 2005). Likewise, three painful cuff compressions, separated by 5 minutes, led to a progressive reduction in the evoked pain intensity (Yarnitsky, 2015). In consequence, if repeated test and conditioning stimuli habituate over time it could have an influence on the CPM-effect during repeated assessments.

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The aim of this study was to measure effects of repeated test-stimuli, with and without parallel conditioning, and to analyse differences between a fixed conditioning and an adapted conditioning paradigm. It was hypothesised that 1) the CPM-effect will be impaired by habituation of the condition stimulus when repeated four times in 20 minutes, and 2) the unconditioned test stimuli will habituate when applied repeatedly.

## **MATERIALS AND METHODS**

### ***Subjects***

Twenty-eight healthy male subjects between 21 – 44 years were recruited (age:  $27.0 \pm 4.8$  years, mean  $\pm$  standard deviation) via social media and advertisements at the university. Exclusion criteria were 1) diagnosis of sleep, neurological, mental or musculoskeletal disorders, 2) pain within the last 48 hours or any use of sleep or pain medication in this period, 3) any history of chronic stress or chronic pain, or skin lesions in the test-areas (calves), and 4) less than 6 hours of sleep over the last 24 hours. All participants received oral and written information about the experiment and gave their verbal and written consent prior to the study. The study was approved by the local Ethics Committee (N-20150065) and was performed in accordance with the Helsinki Declaration.

*INSERT Figure 1: Study design*

### ***Experimental procedures***

The study was designed as two randomised, cross-over experiments (Fig. 1). Each experiment consisted of two sessions: 1) A control-session, and 2) a CPM-session. In the control-session, four 5-min bouts were performed each including two pressure test-stimuli at the dominant leg. Within each bout, the 1<sup>st</sup> test-stimulus was initiated at time 0 and the 2<sup>nd</sup> test-stimulus started 60 seconds after termination of the 1<sup>st</sup> test-stimulus. Each test-stimulus consisted of a ramped pressure stimulation and lasted until the participant reached their tolerance threshold or a maximum of 100 seconds. In the CPM-session, a conditioning stimulus was applied at the non-dominant leg in parallel with the 2<sup>nd</sup> test-stimulus in each bout. To ensure that all participants were exposed to the same amount of conditioning, the conditioning stimuli lasted 104 seconds for all participants. Duration of the conditioning stimulus was set to 4 seconds more than the maximum length of the 2<sup>nd</sup> test-stimulus

to ensure sufficient time for participants to give a real-time verbal pain rating of the conditioning intensity (see below). The intensity of the conditioning-stimulus was set to 70% of the pressure pain tolerance (PTT) intensity assessed on the non-dominant leg. The time between end of pressure stimulation (test-stimulus with or without a conditioning stimulus) in one bout and the start of next bout was no less than 36 s and typically it was approximately 60 s in the conditioning session and 90 s in the control session although this may increase if subjects have low pain tolerance thresholds. There were no additional breaks between bouts.

The two experiments (*fixed vs adapted*) were different in that the adapted included a test-stimulus on the non-dominant leg within each bout (in both control and CPM sessions). In the *fixed* experiment, the conditioning intensity was estimated in the training session (see below) and remained fixed at this level throughout the experiment, whereas in the adapted experiment, the conditioning-stimulus was relative to the test-stimulus on the non-dominant leg in each bout. The sequence of experiments (*adapted, fixed*) and sessions (*control, conditioning*) were randomized among subjects. Experiments were separated by 1-4 weeks and sessions were separated by a five-minute rest period.

For training purpose both experiments included a baseline test consisting of two test stimulations (one on each leg) followed by a CPM-protocol (test stimulus on the dominant leg, conditioning stimulus on the non-dominant leg). The training session was separated from the experiments by a minimum of ten minutes. Data from the training session is not included in the analysis.

### ***Cuff algometry***

Experimental procedures were done with participants positioned in an inclined bed where they also remained during rest. Test and conditioning stimulations were delivered by a computer-controlled cuff pressure algometer (NociTech, Denmark, and Aalborg University), consisting of a computer-controlled air compressor with two independent 7.5 cm tourniquets (silicone high-pressure cuff, VBM Medizintechnik GmbH, Sulz, Germany) (Graven-Nielsen et al., 2017; 2015). The system was connected to an electronic visual analogue scale (VAS, 0-10 cm) and a stop button terminating inflation of the cuffs (Aalborg University, Denmark). Endpoints of the VAS were defined as 0 being

‘no pain’ and 10 cm being the ‘maximal pain’. Cuffs were mounted bilaterally on the most prominent part of the calf and the upper and lower borders of the cuffs were marked on the skin using a permanent marker. These marks were used to visually confirm that the cuffs did not move between the stimulations or sessions.

### ***Test stimulation by cuff algometry***

All test-stimuli were individually adjusted based on constant ramping cuff inflation (1 kPa/s) until subjects pressed the stop button to indicate PTT or until maximum stimulation intensity (100 kPa). During the cuff inflation subjects rated the cuff induced pain intensity on the electronic VAS. For data collection, the pressure equal to 1 cm VAS was considered the pain detection threshold (PDT) whereas PTT was defined as the pressure when subjects stopped the cuff inflation (Graven-Nielsen et al., 2017). If PTT was not reached, 100 kPa was used for statistical analysis.

### ***Conditioning by cuff algometry***

A constant painful cuff pressure stimulus was applied on the non-dominant leg in parallel to the test stimulus and the intensity of the conditioning stimulus was pre-defined as 70% of PTT on the non-dominant leg (Arendt-Nielsen et al., 2010; Rathleff et al., 2016). In case PTT was not reached during the initial training phase on the conditioning leg before the safety limit (100 kPa), the subject was excluded. If subjects reached 100 kPa during the subsequent assessments, to the non-dominant leg during the adapted paradigm, the conditioning stimulus was set at 70 kPa. Pain intensity of the conditioning stimulus was scored by the subjects immediately after the test-stimuli were completed, using a verbal numeric rating scale (NRS, 0-10) with 0 defined as ‘no pain’ and 10 was ‘maximal pain’.

### ***Analysis of conditioning effects***

Pain thresholds are the most common outcome for CPM-effects (Coppieters et al., 2016; Flood et al., 2016; 2017; Naugle et al., 2017; Nir et al., 2011; Razavi et al., 2014; Smith and Pedler, 2017; Zheng et al., 2014) and both PDT and PTT can reliably assess CPM-effects by means of the computerised pressure-cuff (Graven-Nielsen et al., 2017), albeit that within-session reliability of PDT is higher compared to PTT (Imai et al., 2016). In this study, the CPM effects of the conditioning stimulus were analysed by the PDT (for PTT effects see Supporting information) from the test-stimuli (Graven-

Nielsen et al., 2017). CPM-effects are defined as the difference in PDT “during-conditioning” minus “before-conditioning”. As a consequence, a positive CPM-effect reflects that pain thresholds increased during conditioning. Likewise, a positive Control-effect (test-stimulus without conditioning minus the prior test-stimulus) reflects increased pain thresholds for the 2<sup>nd</sup> test-stimuli in the Control session. Four individual CPM-effects and Control-effects were calculated to match each of the four bouts in each session, and finally four netCPM-effect (CPM-effect – Control-effect) were calculated.

### **Statistics**

Results are presented as mean and standard error of the mean (SEM), unless otherwise specified. Pain thresholds and effects were normally distributed (visual inspection of Q-Q plots). To analyse the difference between conditioned and unconditioned test-stimuli absolute values (PDT or PTT) were analysed in a *three-way analysis of variance* (ANOVA) with factors *session* (CPM, Control), *bout* (1-4) and *test-time* (1<sup>st</sup> and 2<sup>nd</sup> test stimulus) as repeated measure. CPM-effects and Control-effects were analysed in *two-way repeated-measures ANOVA* with factors *session* (CPM, Control), and *bout* (1-4). The netCPM-effects were analysed with a *repeated-measures ANOVA* with *bout* (1-4) as factor.

Pain during conditioning (NRS) was analysed with a *two-way ANOVA* with *experiment* as between-group factor and *bouts* as a repeated factor. The intensity of the conditioning stimulus was analysed in a *two-way repeated-measures ANOVA* with factors *experiment* (fixed, adapted) and *bout* (1-4), see supporting information.

Greenhouse-Geisser corrections were applied to all analysis if sphericity was violated. Significant interactions and main effects were analysed in post-hoc with Bonferroni (Bon) comparisons. Effect sizes (*partial eta squared*,  $\eta_p^2$ ) were calculated for CPM-effects and Control effects. Significance was accepted for p-values  $\leq 0.05$ .

## **RESULTS**

Eight of the 28 participants were excluded (age:  $25.5 \pm 3.0$  years); seven subjects did not reach PTT before the cuff algometry safety limit of 100 kPa in the baseline trial, and one participant was unable to participate in both experiments due to work. Thus, if not otherwise mentioned data analysis is



based on 20 participants. During consecutive testing, all PDT-values were below 100 kPa at all time points in both experiments. The time between bouts was 77 seconds on average (see Table 1). Results related to the adaptive experiment can be found in supporting information.

*INSERT Figure 2: Changes in absolute PDT*

### ***Difference between conditioned and unconditioned test-stimuli (CPM- and Control-sessions)***

Analysis of PDT revealed an interaction between *session*, *test-time* and *bout* (Fig. 2a; ANOVA:  $F(3, 57) = 3.17$ ,  $p = 0.03$ ). Post-hoc analysis showed increased PDT in the CPM-session during all the conditioned test-stimuli (Bon:  $p < 0.02$ ), indicating a CPM-effect in all four bouts. In the Control-session, PDT in the 1<sup>st</sup> test-stimulus increased more than the 2<sup>nd</sup> test-stimulus in bout 4 (Bon:  $p = 0.04$ ), and PDT increased in all bouts compared to bout 1 (Bon:  $p < 0.03$ ). Together these results indicate that the repeated stimulations affect the 1<sup>st</sup> test-stimulus in each bout more than the 2<sup>nd</sup>. Between the CPM-session and Control-session differences were found in bout 1, 2 and 4 (#, Bon:  $p < 0.04$ ), where PDT increased in the CPM-session.

Analysis of PTT showed main effects for test-time (ANOVA:  $F(1, 19) = 19.92$ ,  $p < 0.0005$ ), indicating that the 2<sup>nd</sup> test-stimuli were higher than the 1<sup>st</sup>, and *Bout* (Fig. S1a; ANOVA:  $F(1.59, 30.28) = 11.17$ ,  $p = 0.001$ ). The latter showed that PTT increased in all bouts compared to bout 1 (Bon:  $p < 0.02$ ).

*INSERT Figure 3: CPM- and Control-effects*

### ***CPM-effects and Control-effects***

Analysis of PDT showed an interaction between *session* and *bout* (Fig 3a; ANOVA:  $F(3, 57) = 3.17$ ,  $p = 0.03$ ,  $\eta_p^2 = 0.14$ ) and a main effect of *session* was found (ANOVA:  $F(1, 19) = 21.96$ ,  $p < 0.0005$ ,  $\eta_p^2 = 0.54$ ). Post-hoc analysis showed that the change in PDT ( $\Delta$ PDT) was higher in the CPM-session compared to the Control-session for bout 2, 3 and 4 (Bon:  $p < 0.009$ ). In the Control-session,  $\Delta$ PDT dropped from bout 1 to bout 4 (Bon:  $p = 0.03$ ). Together this suggests that over time  $\Delta$ PDT are higher and more constant in the CPM-session compared to the Control-session.

No interactions or main effects indicated a difference in  $\Delta$ PTT between the two sessions (Fig S2a; ANOVA:  $P = 0.11$ ) or over time (ANOVA:  $p = 0.18$ ).

*INSERT Figure 4: Difference between CPM- and Control-effects; a netCPM-effect*

### ***Difference between CPM-effects and Control-effects***

There was a main effect showing an increase in netCPM-effects over time for PDT (Fig 4; ANOVA:  $F(3, 57) = 3.16$ ,  $p = 0.03$ ,  $\eta_p^2 = 0.14$ ), although no pairwise significant differences could be found between individual bouts ( $p < 0.17$ ). No effects were found for PTT ( $p = 0.3$ ).

## **DISCUSSION**

The current study is the first to report that a fixed conditioning stimulation can lead to increased PDT when repeated four times over 20 minutes. The repeated assessments in the Control-session was influenced by habituation to a larger degree than the CPM-session. Contrary to the hypothesis of this study, habituation did not play a significant role during repeated test-stimuli with or without parallel conditioning. The CPM effects were not significant in the adapted conditioning experiment, when compared to the control session, which is probably due to the increase in complexity of the psychophysical protocol. Likewise, the pain tolerance thresholds did not change consistently when compared to a control condition.

### ***Repeated conditioning cause a robust increase in pain thresholds***

A significant increase in PDT and PTT was found in all four bouts during conditioning. These findings are in line with studies using computerized cuff-algometry for both test and conditioning stimuli (Graven-Nielsen et al., 2017; Imai et al., 2016; Petersen et al., 2017) as well as the majority of studies on different combinations of test and conditioning stimuli in healthy subject (Gehling et al., 2016; Granovsky et al., 2016; Nahman-Averbucha et al., 2013; Potvin and Marchand, 2016; Skovbjerg et al., 2017; Vaegter et al., 2015).

### ***Unconditioned (control) test-stimuli also interact***

Repeated, unconditioned painful stimulations are commonly used to study phenomena such as temporal summation of pain (Gehling et al., 2016; Kumowski et al., 2017), and one study used repeated test-stimuli to test for after-effects of a single conditioning epoch (Fujii et al., 2006). No studies have previously evaluated the effects of repeated, individual test-stimuli and compared them to repeated CPM-effects. Over time, painful unconditioned (control) stimuli generally lead to some degree of habituation over time in healthy subjects (Graven-Nielsen et al., 2017) although results are inconsistent across stimulus intensities (Coghill et al., 1993; Fujii et al., 2006; Polianskis et al., 2002; Weissman-Fogel et al., 2015) and interstimulus timing (Lewis et al., 2012).

In this study, the PDT and PTT increased from bout 1 to the last three bouts, and the increase in PDT was greater for the 1<sup>st</sup> compared to the 2<sup>nd</sup> test-stimulus. The increase in PDT over repeated test-stimuli is in line with the findings from Polianskis et al. (2001), reporting that PDT increased over three sequential pressure-cuff stimuli. Another study reported that painful stimuli separated by 30 seconds can lead to transient increases in pain that are normalised after two minutes, leading to systematic changes in pressure-pain threshold from one test-stimulus to the next (Isselée et al., 1997). This could explain why PDT increased more in the four 1<sup>st</sup>-test-stimuli compared to the four 2<sup>nd</sup>-test-stimuli.

It is interesting that the Control-effects were opposite of the CPM-effects, and this could be interpreted to suggest that the 2<sup>nd</sup> test-stimulus is more stable as a consequence of a sequential conditioning by the 1<sup>st</sup> test-stimulus (Yarnitsky et al., 2010). In the fixed-conditioning experiment this could reflect homotopic modulation (Pud et al., 2005). However, this explanation fails to explain the decrease in PDT over time in the adapted-conditioning experiment, which under the same assumptions should be further conditioned, ie. leading to higher PDT. Another possibility is that the Control-session is best understood as the result of the 1<sup>st</sup> test-stimuli leading to a sensitisation of the 2<sup>nd</sup> test-stimulus (Yarnitsky et al., 2015b), possibly centrally and/or peripherally.

### ***CPM-effects and Control-effects***

No other studies have compared repeated CPM-bouts with Control-bouts but four studies have successfully repeated two CPM-bouts in healthy controls within the same session to evaluate reliability (Gerhardt et al., 2017; Julien et al., 2005; Potvin and Marchand, 2016). One study assessed CPM-effects three times using a fixed conditioning intensity (Granovsky et al., 2016; 2017; Imai et al., 2016; Vaegter et al., 2017b). The current study supplement these findings and is the first to show

that a fixed conditioning stimulus can lead to higher PDT and CPM-effects compared to a control-session.

The results from this study complement the repeated CPM-effects found in healthy volunteers (Nahman-Averbuch et al., 2013) and adds that repeated CPM and Control bouts could explore temporal changes in the descending modulatory system in a way, which may be lost when repeated stimuli are only calculated as means (Granovsky et al., 2016; 2017). In addition, the current study make a case for systematic evaluation of repeated, unconditioned test-stimuli (i.e. Control-effects) as a novel supplement to the understanding of pain modulation (Nahman-Averbucha et al., 2013).

### ***A balance between habituation and sensitization***

The difference between CPM- and Control-effects increased significantly over time for PDT in the fixed-conditioning experiment. No changes were found for PTT, which may be related to a ceiling-like effect.

A neurophysiological *dual-process theory of response to repeated stimulation* has been proposed by Groves and Thompson almost 50 years ago (Groves and Thompson, 1970). The theory propose the assumption that habituation and sensitisation rely on separate neuronal mechanisms, which lead to a common behavioral outcome depending on the context. This is almost identical with the current understanding of the CPM-effect being a net-effect of inhibitory and facilitatory mechanisms (Arendt-Nielsen et al., 2017; Yarnitsky et al., 2014). However, interpreting the present data as a dual-process would suggest that PDT in the Control-session 'habituates' over time (Fig. 2a) and that there is a 'sensitisation' occurring during conditioning in the CPM-session (Fig. 2a). As a consequence, the netCPM-effect increases over the four bouts. Future studies could consider the possibility that the netCPM-effect may represent new interpretations of the pro-/anti-nociceptive interactions (Petersen et al., 2016; Vaegter and Graven-Nielsen, 2016; Yarnitsky et al., 2014).

### ***Conditioning pain-intensity and stimulation intensity***

The two experiments in this study resulted in different pressure intensities of conditioning stimuli. This was likely a result of the adjustment to maintain a stable pain report in the adaptive-conditioning experiment (Polianskis et al., 2002). However, the subjective pain rating of the conditioning stimulus did not change as a consequence of the difference in pressure intensity.

The role of conditioning intensity on CPM-effect is not trivial but most studies agree that CPM requires a painful conditioning (Granot et al., 2008; Klyne et al., 2015; Lautenbacher et al., 2002) although inconsistency exists (Honigman et al., 2016). The majority of studies seem to indicate that pain-intensity in itself is not an important constituent for the magnitude of CPM-effects (Granot et al., 2008; Nir et al., 2011; Valencia et al., 2013). However, some studies point towards an association between higher conditioning intensities and increased CPM-effects in single-bout CPM-paradigms (Graven-Nielsen et al., 2017; Izumi et al., 2017; Tousignant-Laflamme et al., 2008) while others have not found no difference in CPM-magnitude between moderate and intensely painful conditioning (Nir et al., 2011). In line with previous studies, the current data supports that painful conditioning stimuli lead to CPM-effects, and adds that a fixed conditioning intensity can be used to measure repeated CPM-effects.

Previously, a study on healthy subjects have identified time-dependent changes in pain during repeated cuff-stimulations (Polianskis et al., 2001), and several studies have identified habituation to electrical and heat-pain stimulations (Bingel et al., 2007; Greffrath et al., 2007; Hollins et al., 2011; Milne et al., 1991; Staud et al., 2014; Treister et al., 2010). The mechanism behind habituation to repeated stimuli is not well understood. Some authors suggest that the opiodergic system plays a role in habituation (Bingel et al., 2007; Ernst et al., 1986) while others find that habituation may be independent of the opiodergic system (Rennefeld et al., 2010).

### ***Limitations***

The current study involved only healthy, male participants and caution must be taken before extrapolating to the general population as well as to both genders. The duration of each bout was set at 300 seconds, which is the minimum time need to organise the computerised CPM-session. The fact that subjects were instructed to stop the test-stimuli once it reached their PTT meant that subjects reaching the PTT before 100 kPa (i.e. 100 seconds) would also have longer time without stimulation (before the next bout) compared to individuals who reached 100 kPa. As a consequence, it is likely that the risk of accumulating effects was higher in individuals reaching 100 kPa. It is also possible that the duration of the break between sessions or the individualised intensity of the conditioning stimuli, could play a role. The cross-over design, application of a control-session and the use of a computer-controlled pressure-cuff algometer provides methodological gains to this study. The control-effects, found in this study, should be tested in larger samples of both genders. The reproducibility of consecutive CPM-effects needs to be validated in clinical samples of both genders.

## **Conclusion**

This study found that conditioned test-stimuli significantly increase pain thresholds in four consecutive 'CPM' bouts, and is the first to show that the increase in pain thresholds is higher than repeated, unconditioned test-stimuli. Furthermore, CPM-effects are unchanged over time, which contrasts to Control-effects that decrease over time. Moreover, repeated unconditioned test-stimuli may be influenced by local facilitatory effects, which apparently are reduced when using the conditioning protocol and activation of descending modulatory mechanisms. A clinical implication of this study is that the CPM-sessions and Control-sessions together could be a novel tool to explore the relationship between the facilitatory and inhibitory systems in humans.

## **AUTHOR CONTRIBUTIONS**

All authors contributed to the conception and design of the study as well as making intellectual contributions to its content. MH, KKP and TGN contributed to the analysis and interpretation of the data and drafting of the manuscript. All authors discussed the results, commented on and approved the final manuscript.

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## TABLES

**Table 1.**

TABLE S1		Bout 1	Bout 2	Bout 3	Bout 4
CPM-session	1 <sup>st</sup> TS	17	17	17	18
	2 <sup>nd</sup> TS	18	17	18	17
Control-session	1 <sup>st</sup> TS	18	16	17	17
	2 <sup>nd</sup> TS	19	17	16	17

**Table 1:** Number of participants (total n = 20) reaching PTT on the test leg prior to the limit of 100 kPa. Abbreviations: Test-stimulus (TS), Conditioned Pain Modulation (CPM).

The duration of each bout was individually adjusted and depended on PTT, meaning that participants with higher threshold had less time between the bouts. The time between bouts in the fixed experiment was  $67.9 \pm 5.1$  s (CPM-session) and  $97.4 \pm 10.9$  s (Control session).

## FIGURE LEGENDS

**Figure 1.** Participants (n = 20) were randomised into this cross-over study with two experiments (fixed and adapted conditioning). **(a)** Both experiments consisted of two sessions (CPM and Control) consisting of four bouts. **(b)** Each bout included two painful test-stimuli (shown in blue) on the dominant leg (1<sup>st</sup> and 2<sup>nd</sup>). In the CPM-bouts a conditioning stimulus, which was applied to the non-dominant leg (shown in red). In the adaptive-conditioning experiment (not shown) an additional test-stimulus on the non-dominant leg was applied between 1<sup>st</sup> and 2<sup>nd</sup> test-stimulus (CPM- and Control-bouts).

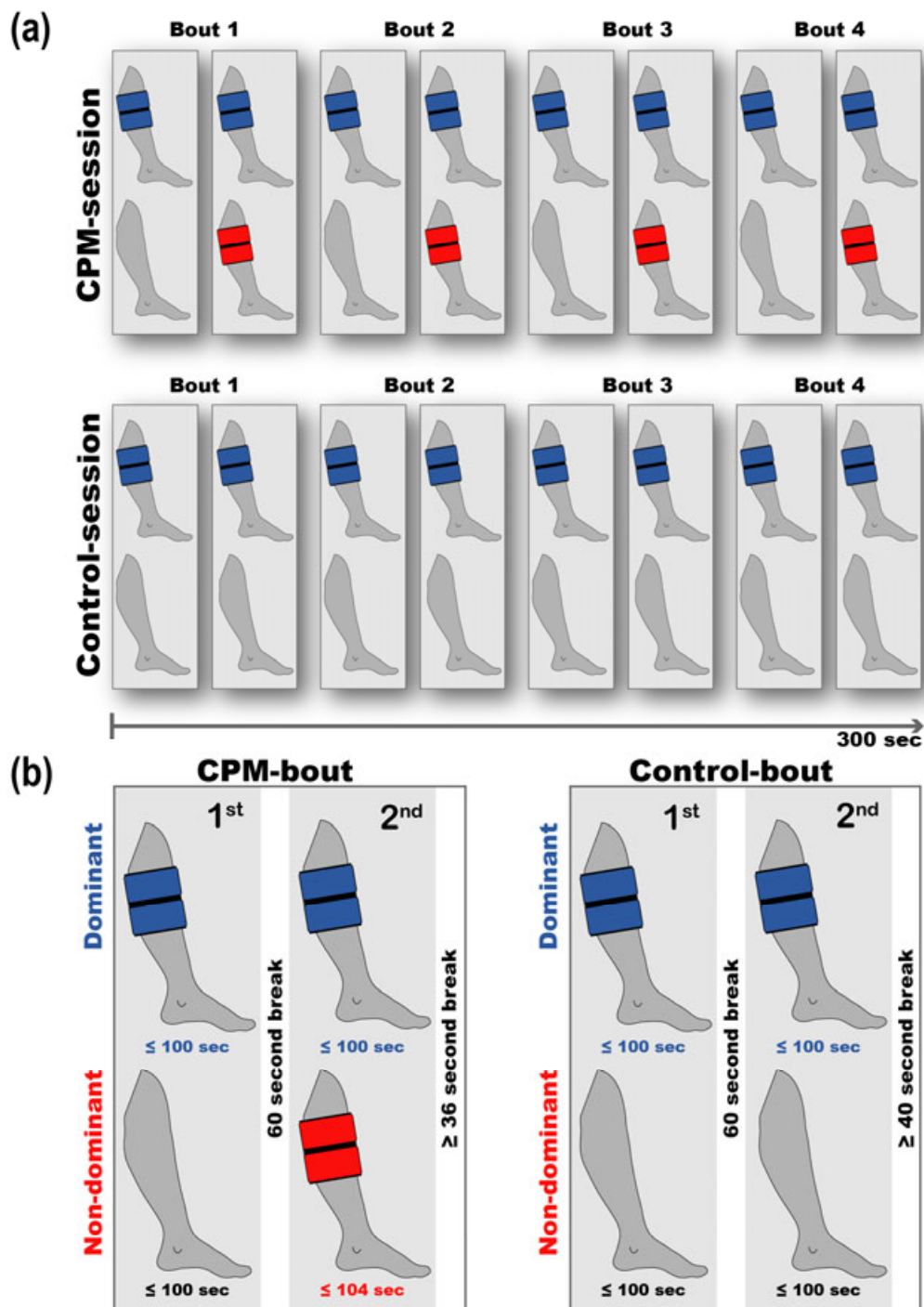


**Figure 2.** Mean ( $\pm$ SEM,  $n = 20$ ), (a) pain detection thresholds (PDT) and (b) pressure-pain tolerance threshold (PTT) in the 1<sup>st</sup> (unconditioned) and 2<sup>nd</sup> (conditioned) test-stimuli for each bout. Each bar show PDT/PTT of the eight test-stimuli (1<sup>st</sup> in open bars and 2<sup>nd</sup> in black bars) in each of the two sessions (CPM and Control). **(a)** PDT increased during the 2<sup>nd</sup> (conditioned) test-stimulus compared to the 1<sup>st</sup> test-stimulus (\*, Bon:  $p < 0.02$ ) as well as to the corresponding test-stimulus in the Control-session (#, Bon:  $p < 0.04$ ). Within the Control-session PDT increased in all bouts compared to bout 1 (§, Bon:  $p < 0.03$ ). **(b)** PTT during the 2<sup>nd</sup> test-stimulus was higher than the 1<sup>st</sup> (\*,  $p < 0.0005$ ). Furthermore, PTT in the three last bouts were higher compared to PTT in the first bout (§,  $p = 0.02$ ).

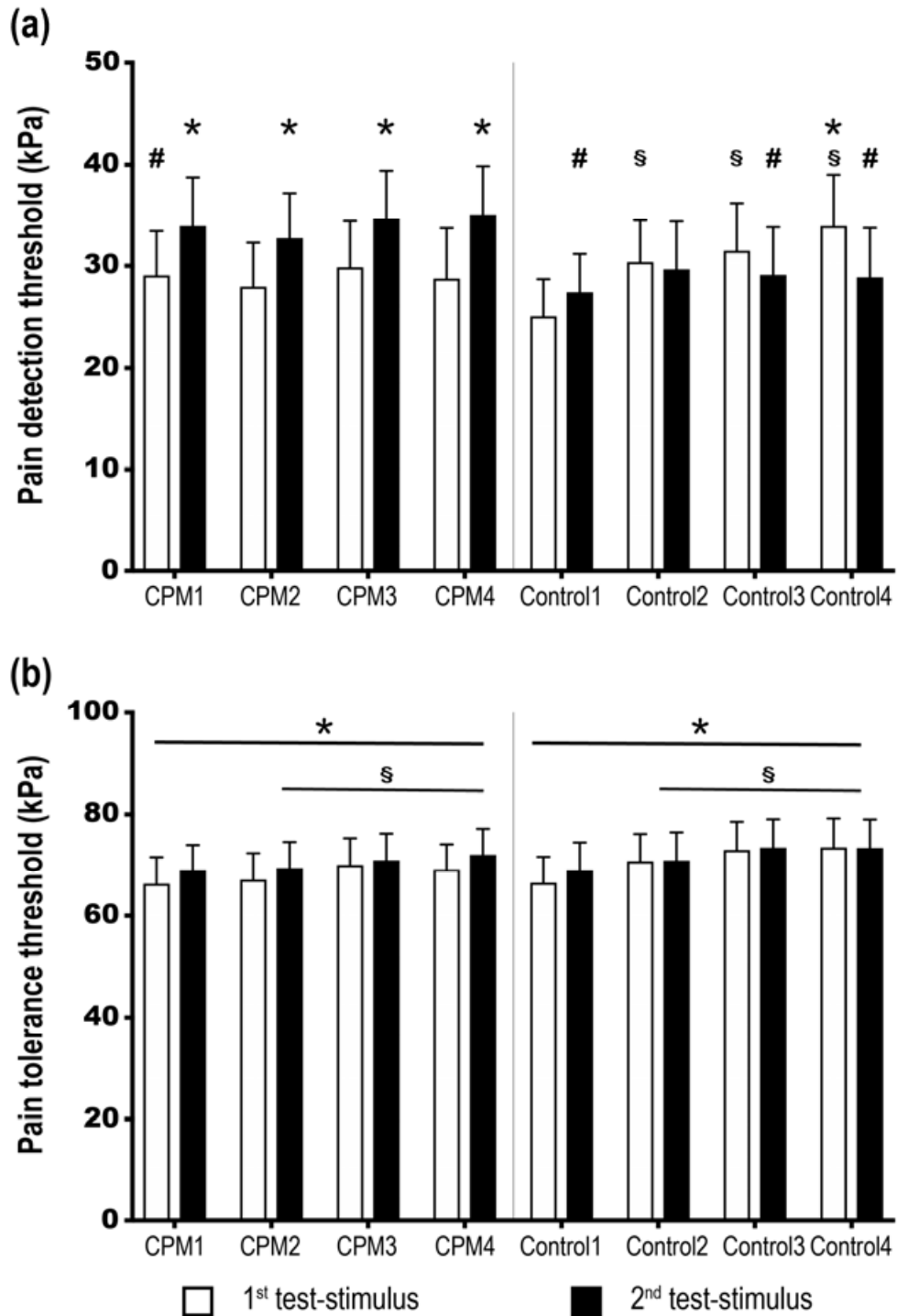
**Figure 3.** Mean CPM-effects and Control-effects ( $\Delta$ PDT;  $\pm$ SEM,  $n = 20$ ) in the CPM-session (black circles) and Control-session (open circles), respectively, for **(a)** pain detection threshold (PDT) and **(b)** pressure-pain tolerance threshold (PTT). **(a)** CPM-effects were higher than the time-wise comparable Control-effects (\*, Bon:  $p < 0.009$ ) and the Control-effects decreased from bout 1 to bout 4 (§, Bon:  $p = 0.03$ ). **(b)** There were no differences between CPM- and Control-effects for PTT.

**Figure 4.** The difference between the CPM-effect and Control-effect (netCPM-effect) represents the relative balance between habituation and sensitisation and shows an increase in PDT over time (\*,  $p = 0.03$ ).

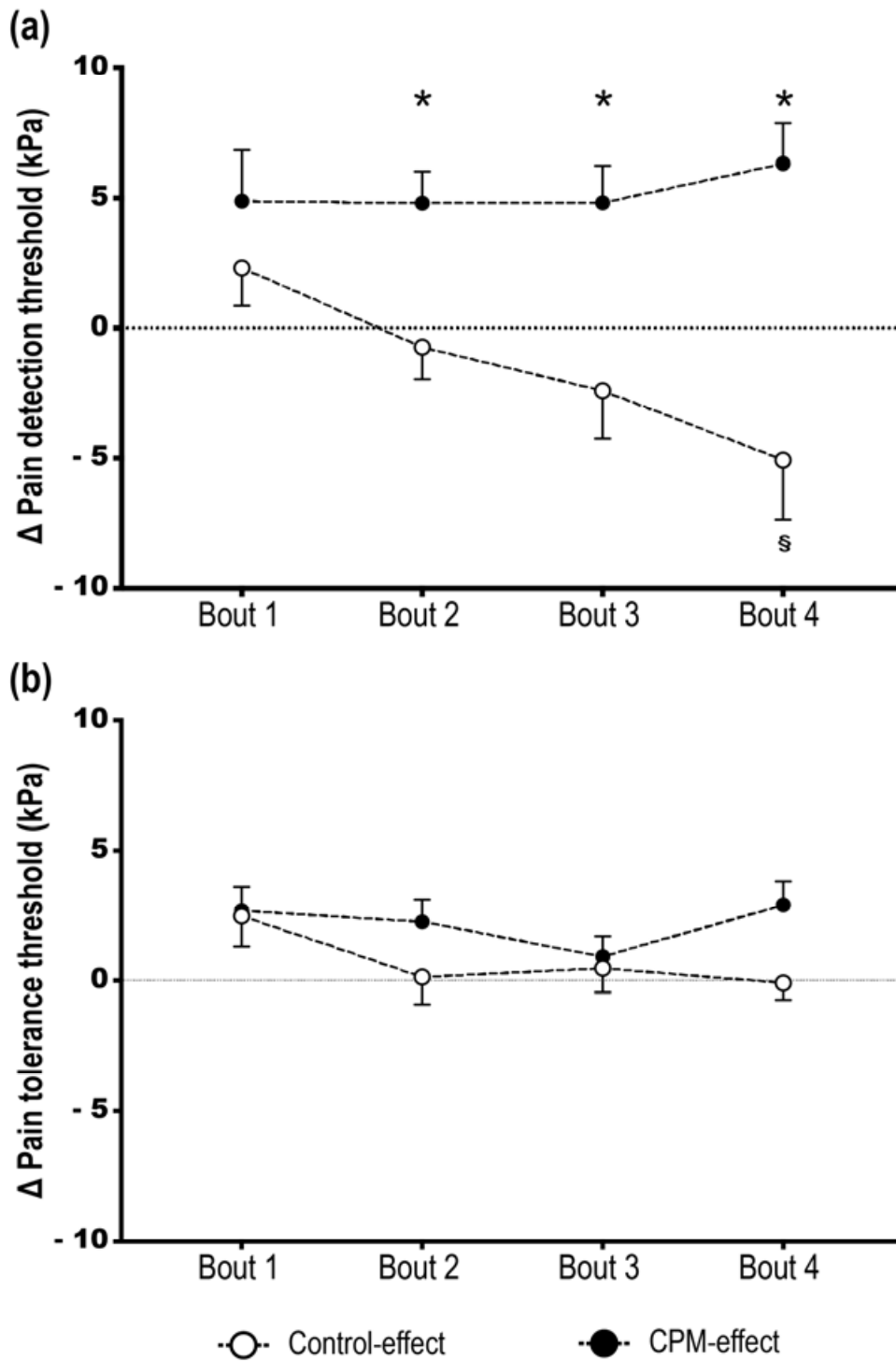
**Fig. 1 Study design**



**Fig. 2 Conditioned vs unconditioned test-stimuli**



**Fig. 3 CPM- and Control-effects**



**Fig. 4** **netCPM-effect**

