Assessment of Changes in Laryngeal Configuration and Voice Parameters Among Different Frequencies of Neuromuscular Electrical Stimulation (NMES) and Cumulative Effects of NMES in a Normophonic Subject: A Pilot Study

Pedro Amarante Andrade, Ph.D., Marek Frič, Ph.D., and Zdeněk Otčenášek, Ph.D., *Prague, Czechia*

Summary: Introduction. Neuromuscular electrical stimulation (NMES) is a complementary resource to voice therapy that can be used for the treatment of hypofunctional voice disorders. Although positive clinical studies have been reported, neutral and even potentially harmful effects of NMES are also described in the literature. Furthermore, in the studies examined by the authors, the use of different methods of NMES have been identified, which further contributes to the inconsistent results found among studies. Moreover, limited rationale is provided for the chosen NMES parameters such as electrode placement, frequency of NMES and length of treatment. The aims of this pilot study were to investigate the a) impact of different frequencies of NMES on glottal configuration and vocal fold vibration patterns and b) changes in laryngeal configuration and vocal output across 12 minutes of NMES.

Method. Three experiments were carried out looking at changes in laryngeal configuration and voice output using different imaging techniques (fibreoptic nasolaryngoscopy and high-speed video), acoustical analysis (F0, formant analysis, SPL, CPPS and LHSR values), electroglottography (EGG) and Relative Fundamental Frequency (RFF) analyses. Glottal parameters and acoustical measures were recorded before, during, and after stimulation. Data was collected at rest and during phonation.

Results. Overall the results showed global changes in laryngeal configuration from normal to hyperfunctional (ie, increased RFF, SPL, CQ, and stiffness). Changes were more pronounced for lower frequencies of NMES and were significant within less than three minutes of application.

Conclusion. NMES is an effective resource for the activation of intrinsic laryngeal muscles producing significant levels of adduction within few minutes of application. Lower NMES frequencies produced greater muscle activation when compared to higher frequencies.

Key Words: Neuromuscular electrical stimulation—NMES—TENS—Voice therapy—Voice treatment—Electrical stimulation−Laryngeal stiffness—Relative fundamental frequency.

INTRODUCTION

The most common cause of glottic insufficiency (GI) is vocal fold paralysis (glottal inability to adduct) and paresis (partial inability to adduct).[1,2] They are associated with failure of the recurrent and/or the external division of the superior laryngeal nerves, both part of the vagus nerve, to activate the extrinsic muscles of the larynx and can be presented uni or bilaterally. The most frequent etiology of vocal paralysis and paresis is iatrogenic injury after surgery.[3] Three types of nerve injury were described by Seddon 1943[4] a) *axonotmesis*, injury to the peripheral nerve with no or only partial damage to the muscle b) *neuropraxia*, muscle paralysis without nerve degeneration, or c) *neurotmesis* which is a complete division of the nerve. Regardless of the type of nerve injury, spontaneous recovery rarely occurs beyond 18 months' from the onset of the paralysis, with the highest probability of spontaneous recovery occurring between 1.5 to 6 months'.[5,6] In specific, spontaneous recovery of unilateral vocal fold paralysis only occurs in approximately 40% of cases.[7] Once a muscle is denervated, it eventually becomes atrophic with complete absence of muscle fibers, despite the presence of functioning synapses which may still be intact.[8] Therefore, it is essential that treatment for vocal fold paralysis or paresis should be made available early, within a few months of onset.

In general, vocal fold paralysis and paresis are treated with speech/voice therapy or surgery, with the most common surgical procedures being injection augmentation, medialization thyroplasty[9] or recurrent laryngeal nerve anastomosis.[10] The aim of surgery is to improve the GI by medializing the position of the paralyzed vocal fold. Although surgical intervention improves voice quality, it rarely produces normal voices.[11] In addition to this, a compromised airway passage, late extrusion of the prosthesis, and delayed hemorrhage are all possible negative consequences of medialization surgery.[12−14] Considering this, alternative methods such as electrical stimulation (ES) of the paralyzed muscles/nerves seem to be a good alternative to surgical intervention.

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From the Music and Dance Faculty, Academy of Performing Arts in Prague, Musical Acoustics Research Centre, Prague, Czechia.

Address correspondence and reprint requests to Pedro Amarante Andrade, Academy of Performing Arts in Prague, Musical Acoustics Research Centre, Malostranské nám. 258/13, 118 00 Prague 1, Prague, Czechia. E-mail addresses: pedro.andrade@hamu.cz marek.fric@hamu.cz zdenek.otcenasek@hamu.cz

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ES applied to limb muscles has been shown to increase a) the content of muscle contractile proteins;[15] b) number of enzymes used in aerobic pathways,[15] c) muscle contraction and relaxation times;[16,17] d) resistance level to fatigue,[18,19] e) mitochondrial size and f) capillary density and blood supply.[20]

For voice treatment, early studies using ES, in specific functional electrical stimulation (FES), have been successfully carried out with pacemaker implants that activate the posterior cricoarytenoid muscles (PCA) for cases where the paralyzed vocal fold remained in the medial position.[21–24] Stimuli were used to produce abduction during inspiration in canine samples[21,25] and subsequently used with human subjects.[26–28] Although this procedure retains the original laryngeal configuration, which is not the case with injection augmentation and medialization thyroplasty, it is invasive, as the electrode array needs to be in direct contact with the PCA muscles. ES can also be delivered using small needles that penetrate the skin (ie, percutaneous ES). This method provides great results as electric current is delivered closer to the targeted structure and therefore requires lower intensity levels; however, it is considered a minimally invasive procedure with possible side effects such as bruising and bleeding.[29] Therefore, as an alternative, using surface electrodes for ES is preferable. Although ES using surface electrodes may be hindered by skin and subcutaneous fat impedance, it does not require piercing the skin and therefore can be performed by trained non-medical specialists. Due to this advantage, most recent treatment modalities using ES for voice patients have been conducted using surface electrodes.

Primarily, all ES techniques involve the use of electricity to activate the motor end plates in the muscles or neural pathway in order to cause muscle contraction. Many ES methods have been developed, receiving different names according to their modality (eg, EMS = Electrical muscle stimulation, NMES = Neuromuscular electro stimulation, TES = Transcutaneous electrical stimulation, TENS = Transcutaneous electrical nerve stimulation, PES = percutaneous electrical stimulation). Muscular and neural determine the targeted structure, whilst transcutaneous (through the intact skin), and percutaneous (through the skin using a needle) refers to the delivery method. In addition to this, the term functional is used when the ES is carried out during muscle activation or physical activity (eg, FES). The types of stimuli used for ES vary in amplitude, frequency of stimulation (Hz), wave type (eg, quadratic, sawtooth), phase (ie, biphasic or monophasic), phase duration or pulse length. The combination of the above-mentioned parameters will determine the level and type of stimulation and can therefore be aimed at muscle strengthening or pain relief. As a consequence of possible application of ES for opposite aims, two major types of ES techniques are used for voice disorders: TENS, which is considered non-invasive as lower intensities (with frequency of activation normally set at 10 Hz) are used, aims to produce muscle relaxation and pain relief for individuals with hyperfunctional voice disorders; and NMES or TES (henceforth referred to as NMES) that uses higher intensities and frequencies (usually at 80 Hz) aimed at muscle strengthening for individuals with hypofunctional voice disorders.

TENS and NMES techniques can be performed with the same device assuming that it allows for controlling the stimulation parameters. Manipulating changes in intensities is relatively straightforward as levels of intensity will determine the level of muscle activation, because more muscle fibers will be recruited with increased intensities. On the other hand, understanding the rational that underpins the changes in frequency used for ES may not be as intuitive for voice professionals as expected. When dealing with soundwaves, frequency, and period are reciprocal (ie, higher frequencies produce shorter periods) and interdependent (ie, changes in frequency affects period and vice versa). These changes are relevant to application, for example, low frequency (longer periods) ultrasound allows for deeper tissue penetration whilst higher frequencies with shorter periods are used for superficial application.[30] However, changes in frequency for ES do not necessarily work in the same way. ES uses predetermined phase durations. Which is why the term pulses per second (pps) is often used instead of frequency. A graphical representation of the relationship among phase duration, frequency, and intensity is given in figure 1.

Normally, the cycle profile for a single pulse (top row) presents two phases (alternating current), separated by an interphase interval, and therefore is referred to as a biphasic waveform. Consequently, the period is determined by the duration of the two phases (e.g., 0.2 ms for TENS) and the interphase interval (eg, 0.1 ms). Changes in frequency and intensity (second row) are independent from the phase duration. Consequently, raising the intensity, frequency, and phase duration of stimulation was found to increase muscle contraction[31,32] due to action potential summation, eventually leading to muscle tetany. Overall, TENS uses shorter phase durations (0.05-0.2 ms with 0.2 ms commonly used for voice therapy) at lower frequencies,[33] whilst 0.3 ms, and above at higher frequencies is recommended for NMES.

To better appreciate the difference in duration of activation in voice therapy between TENS and NMES, the total length of stimulation per second can be calculated by multiplying the phase duration (biphasic waveform) by the frequency (eg, TENS: 0.2 *2 *10 = 4ms/s, figure 1 left column). A similar calculation for NMES using the VitalStim Therapy Specialty Program[34] (ie, a swallowing therapy protocol using NMES with the VitalStim device) which uses 80 Hz with 0.3 ms phase length, will result in 48 ms/s of stimulation (Figure 1 right column). The 12-fold higher length of activation used for NMES compared to TENS for voice therapy is further increased by the use of much smaller electrodes in NMES, which concentrates the electric charge in the body region immediately in contact with them. Additionally, work time, resting time, and ramp time (in seconds) can also be controlled. These refer to the dynamics of ES delivery where breaks between periods of stimulation are considered in order to allow muscle

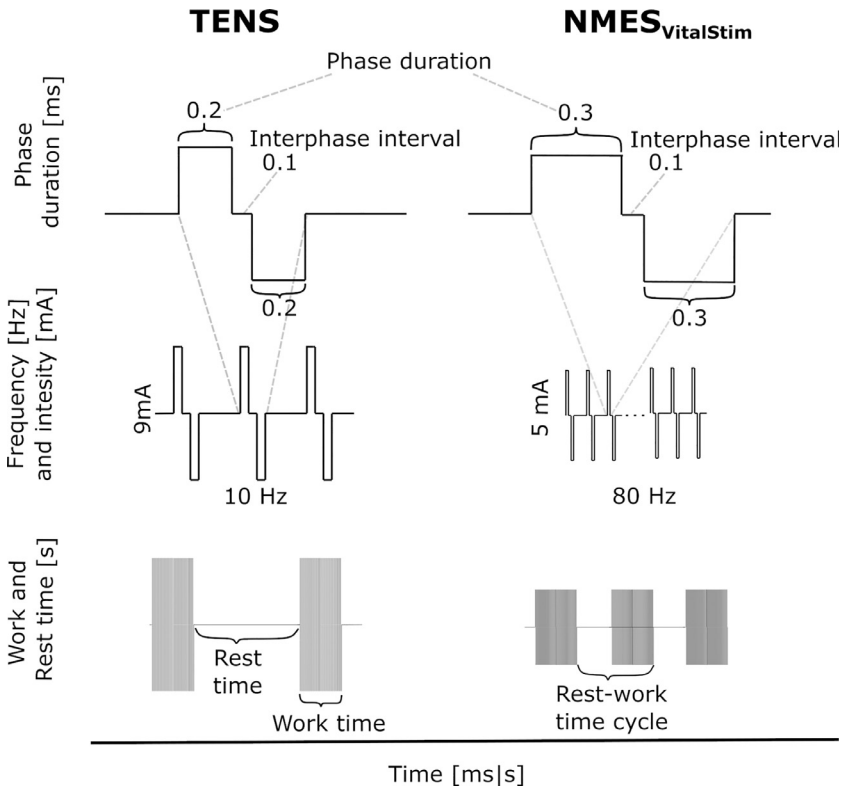

FIGURE 1. Illustration of different TENS and NMES parameters commonly used for voice therapy.

repolarization (Figure 1 lower row). In clinical practice, changes to ES parameters may be used to customize therapy. Feiner et al,[35] when aiming to reduce discomfort during NMES application, suggested raising intensities whilst reducing phase duration in order to shorten the length of stimulation.

Depth of penetration is an important issue for ES of the intrinsic laryngeal muscles as the electrical current must pass through multiple structures (skin, adipose tissue, platysma and strap muscles, and cartilage) before reaching their targeted muscles. Some studies have demonstrated that the limitations of intrinsic muscle activation using ES can be attributed to low levels of current penetration.[36–38] In specific, concerned with airway protection, Humbert et al 2008,[38] looked at vocal fold adduction levels during NMES and found a small change in vocal fold anterior angle after intervention. This led to their conclusion that the changes were not clinically relevant for dysphagic patients.

TENS

Due to the less invasive characteristics of TENS, it has recently become a more common modality of ES, specifically for subjects with pain related issues such as muscle tension dysphonia.[39–44] TENS produces pain relief (analgesia) via opiate release.[45] An alternative method for pain relief for patients with muscle tension dysphonia uses TENS at higher frequencies (100Hz).[43] At higher frequencies TENS produces paresthesia (ie, tingling sensation) which is linked to the activation of more superficial non-painful receptors. The physiology of high frequency TENS is supported by the gate control theory of pain which advocates that non-painful stimuli close to pain receptors prevent the pain sensation from travelling to the central nervous system.[46]

In voice therapy, TENS are usually applied to the trapezius muscles as well as the frontal part of the neck. Due to the larger pads used in TENS, no specific intrinsic muscles are targeted. Recent studies have shown positive results for TENS with dysphonic patients, among them improved phonation comfort,[40–44,47] lowered frequency, and intensity of musculoskeletal pain in the neck and shoulders,[40,42–44,48] improved voice quality,[39,42,43,47] in specific improved vocal stability[48] and glottal closure.[41]

NMES

In order to produce muscle contraction and strengthening, frequencies between 35 to 80 Hz at relative high amplitudes are normally used in NMES. Therefore, NMES is considered more invasive than TENS. The application of NMES aims to prevent atrophy of paretic muscles, aid regeneration of damaged muscular tissue and prevent fibrillation (ie, rapid, irregular, and unsynchronized contraction of muscle fibers),[49,50] hence increasing muscle activity and therefore applicable for use in hypofunctional conditions. NMES has shown good results for individuals with swallowing problems, with studies supporting the use of the technique as an additional resource for therapy.[51–59] Whilst others suggested caution due to possible adverse effects, such as the activation of non-targeted structures.[36,38,60]

In voice therapy, NMES is usually applied at the front of the neck and aims to activate intrinsic laryngeal muscles. Guzman et al 2014[61] found that NMES aimed at the cricothyroid muscles for patients with superior laryngeal nerve weakness produced reduction in breathiness, increased vocal range, better control over the passaggio and reduction in voice complaints. Ptok and Strack 2008[62] found that NMES reduced vocal fold vibration irregularity for patients with unilateral recurrent laryngeal nerve paresis. LaGorio et al 2010[63] found that NMES reduced vocal fold bowing, producing improved acoustic laryngeal, physiologic, and patient-centered positive outcomes. Conversely, some studies found no significant changes in voice acoustics using NMES.[64,65] For example, Gorham-Rowan et al 2010,[64] following the VitalStim protocol (ie, One-hour long sessions using 0.3 ms phase length at 80 Hz), found no significant changes in fundamental frequency (F_0), jitter, shimmer, signal-to-noise ratio or loudness after intervention. In addition to this, the authors highlighted the possible side-effects to this technique as confirmed by delayed-onset muscle soreness after a 1-hour long session of NMES with normophonic subjects. Fowler et al 2011,[65] also following the VitalStim protocol, found significant changes in F_0 and relative sound level after NMES, however with variable direction and magnitude. The authors confirmed delayed-onset muscle soreness after 30 and 60 minute-long NMES sessions.[65,66] Apart from Ptok and Strack 2008,[62] all NMES studies for voice therapy used the VitalStim device with 80 Hz and 0.3 ms phase length for at least 30 minutes of application. These NMES settings were likely used due to the fixed pulse rate and phase duration parameters in the VitalStim model 5900,[67] which was used in most voice studies. In addition to this, it seems that the 60-minute long NMES sessions, also used in most of the voice studies, was chosen based on the VitalStim protocol for swallowing treatment. Intensity, electrode placement and sizes varied among studies. These may be contributing factors to the somewhat conflicting results found in the literature regarding the use of NMES for voice stimulation. Additionally, as most voice studies focus on acoustical analysis, evidence of the effects of NMES on glottal behavior and laryngeal configuration is still lacking.

In this investigative pilot study, three experiments were conducted to provide a visual assessment of laryngeal changes with NMES supported by acoustical analysis. In the first experiment, we aimed to quantify laryngeal muscle activation at different frequencies of NMES in order to compare their effectiveness in activating intrinsic laryngeal muscles. In the second and third experiments, we looked at changes in laryngeal configuration and vocal output across a 12-minute long NMES session to better understand cumulative effects. In our investigation, a novel configuration of the electrodes was used. Smaller electrode sizes were chosen to better concentrate the electric current along the targeted area. Similar electrode poles were placed at the origin of the cricothyroid muscles (at the cricoid cartilage) and at the posterior part of the inferior margin of the thyroid cartilage respectively. This configuration aimed at accessing the intrinsic laryngeal muscles via the cricothyroid space which is absent of cartilaginous tissue and therefore expected to allow better penetration of the electric current. Fiberoptic nasolaryngoscopy and high-speed videoendoscopy recordings were used during phonation and normal respiration of a normophonic subject. This study is a precursor to a larger clinical study which aims to assess changes in laryngeal activation using NMES for clients with vocal fold paralysis/paresis. The results from this study will be used to inform the application of NMES in the clinical setting.

METHOD

In order to ascertain the impact of NMES on laryngeal configuration and voice quality, three separate experiments were conducted. *Experiment one* looked at the influence of different NMES frequencies on glottal configuration and vocal fold vibration patterns; *experiment two and three* assessed the cumulative effects of NMES on laryngeal configuration and voice quality, respectively, during a 12-minute-long NMES session.

Participant

Due to the intrusive nature of this investigation, a single subject study design was used where the author (PA) attended three NMES sessions where fiberoptic nasolaryngoscopy and high-speed videoendoscopy (HSV) were used. Two other volunteers were recruited for the study however they were not well suited for the long endoscopic session required in this study. The experiments were carried out at one-week intervals to avoid carry over effects and to provide enough time for any potential symptoms of delayed-onset muscle soreness to be resolved.

NMES parameters

In all three experiments, NMES was performed using the VitalStim Plus device (Chattanooga Group, Chattanooga, Austin, TX, USA) which allows changes in the stimulation parameters including frequency and phase duration. A co-contraction method was chosen with the phase duration set to 0.3 ms with 3s work time, 5s as rest time and 1s ramp time. VitalStim adult size bipolar electrodes (VitalStim REF 59000; Chattanooga Group, Chattanooga, Austin, TX, USA) were used, however due to the limited space across the stimulated area as well as the likely possibility of contact between electrodes, the electrodes size was reduced from 2.1 to 1 cm in diameter. Similar poles were placed at the origin of the CT muscles and posterior part of the inferior margin of the thyroid cartilage respectively (Figure 2) . This configuration was used with the aim to access the intrinsic laryngeal muscles of the larynx via the lateral extension of the cricothyroid space. Electrodes were held in place using a highly adhesive medical tape. Conductive gel was used to improve contact between electrodes and skin.

In experiment 1, the NMES intensity was gradually increased by 0.5 mA until a strong feeling of grabbing sensation was perceived by the tested subject as instructed in the VitalStim guidelines. The amplitude level of 5 mA was then kept during all 3 experiments.

Procedure and task

Experiment 1 - Assessment of laryngeal muscle activation across different frequencies of NMES using accelerometer and fiberoptic nasolaryngoscopy.

The tested subject sat comfortably in a chair with a custom-made neck support device placed immediately below the chin. The support was used to provide comfort and reduce head movement during data collection. The NMES frequencies assessed were 5,10,15,30,60 and 80 pps. Fiberoptic nasolaryngoscopy was performed using an ORL-Vision RS1 (orlvision GmbH, Lahnau, Germany) with a Highlight Plus Invisia Stroboscope (equivalent to 180W xenon lamp) (Invisia, Padova, Italy). The default frame rate of the ORL-Vision RS1 was used at 24 fps. With the aim of minimizing changes in position and distance of the endoscope's lens from the glottis, the head of the endoscope was attached to a mini microphone stand and placed directly in front of the subject's head. Glottal area (GA) values were obtained for two consecutive NMES rest-work cycles (refer to figure 1) using the Glottal Analysis Tools 2020 software (GAT) (University Hospital Erlangen, Erlangen, Germany). In order to obtain GA values during normal breathing (with and without NMES), the GAT's manual segmentation mode was used. As GA values were calculated only during normal breathing, it directly relates to glottal adduction. Accelerometer (ACC) (PCB Electronics 352C23, 1-Channel, battery-powered, ICP sensor signal conditioner 480E09) and audio signals were also recorded. The audio signal was only used for annotation during the experiment. Throughout the experiment, the tested subject's hearing was masked using a noise cancelling headphone MPOW H19 IPO (MPOW Technology Co., Shenzhen, China) playing loud classic music. Masking was done in order to limit the subject's ability to counteract changes in F_0 caused by the activation of the CT muscles due to NMES. The subject confirmed the inability to hear his own voice and was not able to answer questions uttered by the investigator standing away from the subject's visual field. In experiment one, the subject's task was to remain still and breathe normally whilst at least two rest-work time cycles for each NMES frequency were recorded.

Experiment 2 - Assessment of changes in laryngeal configuration using traditional and high-speed video endoscopy during a 12-minute long NMES session.

In experiment two, high-speed videoendoscopy (HSV) was recorded using a VisionResearch Phantom V611 (VisionResearch Phantom, New Jersey, USA) with an Olympus CLV-S45 (300W) light source (Olympus Corporation, Tokyo, Japan). The VisionResearch Phantom V611 camera was mounted on a tripod and the rigid endoscope set at a comfortable height and angle for the tested subject. The tested subject was standing directly in front of the reference monitor in order to visually manage the laryngeal view. To aid management of the laryngeal view and produce laryngeal images with similar sizes, stickers were placed on the monitor marking the anterior and posterior angles of the glottis and used as a target position for the glottis. In addition to the HSV recording, a traditional video recording (at 10 fps) of the laryngoscopy session was simultaneously obtained using the OBS screen recording software (Open Broadcaster Software, GNU compiler collection internals, Boston, USA)[68] yielding two sets of imaging data. The HSV data was primarily used to analyze changes during phonation, whilst the screen recording was used to assess changes in laryngeal configuration during the NMES session. ACC and audio signals were obtained according to experiment 1. Electroglottography (EGG) signal was also recorded using a Laryngograph A-100 device (Laryngograph, Wallington, UK) (Figure 2). With the aim of avoiding damage to the VitalStim Plus or Laryngograph A-100 devices, they were used alternately. As both the EGG and NMES electrodes were fixed to the larynx, the EGG electrodes were disconnected from the Laryngograph A-100 during NMES and the VitalStim Plus switched off during EGG recording. The VitalStim Plus frequency was set to 35 pps as it produced a comfortable and smooth muscle activation. A 12-minute session of NMES was used to investigate changes in laryngeal configuration across time. HSV recordings of sustained phonation were obtained every three minutes during the NMES session as well as before and after, producing five points of assessment (ie, 0,3,6,9 and 12 minutes). During NMES the subject was advised to remain still and breath normally. Auditory masking was performed as per experiment one. Due to the presence of the rigid endoscope, only the vowel /i/ was used for the HSV recording. Image analysis was performed using the GAT and the ImageJ (National Institutes of Health, Bethesda, MD, USA)[69] software.

Experiment 3 - Acoustic analysis during a 12-minute long NMES session.

The acoustic analysis was carried out in a sound treated room. The subject sat comfortably whilst audio and EGG data were collected. EGG and VitalStim data collection management was carried out as per experiment two. Acoustic data was obtained using a Sennheiser ME 62 microphone (Senheiser, Wedemark, Germany) placed at 30 cm from the mouth and analyzed using the Praat software (Praat: doing phonetics by computer, version 6.1.35, Amsterdam, The Netherlands, www.praat.org).[70] The sustained vowels /a/, /i/, /u/ were recorded. The subject was advised to produce all utterances at his habitual pitch whilst remaining relaxed. Fundamental frequency (F_0), smoothed cepstral peak

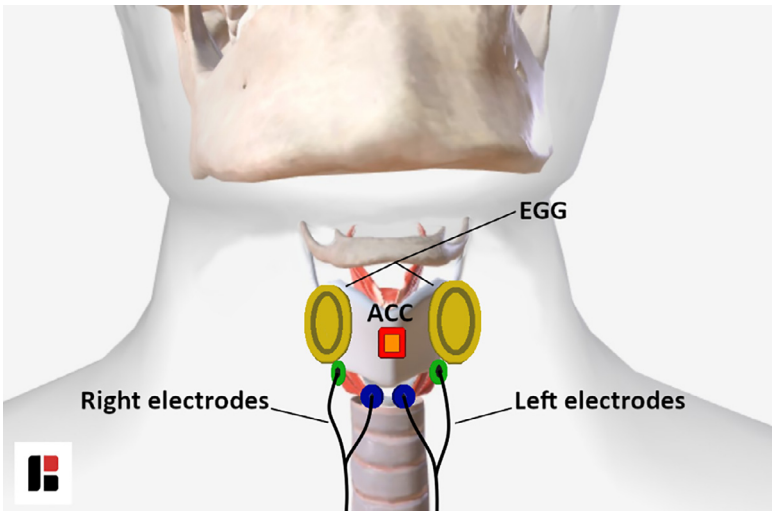

FIGURE 2. Combined configuration of electrodes and accelerometer placement for the 3 experiments. Blue (CT origin) and green (posterior part of the inferior margin of the thyroid cartilage) NMES electrodes for the left and right channels respectively. Electroglottographic (EGG) electrodes in yellow. Accelerometer (ACC) (red and orange) was fixed between the EGG electrodes. All components were fixed onto the neck using a thick medical surgery tape (not shown in the figure). Image adapted from Biodigital (http://www.biodigital.com). (Color version of the figure is available online.)

prominence (CPPS), low/high spectral ratio (LHSR = frequencies < 4000 Hz/ > 4000 Hz) and sound pressure level at 30 cm from the mouth (SPL) were analyzed. Only relative SPL values produced by the Praat software were used in this analysis, therefore the results cannot be directly compared to other studies. FFT was used to assess changes in formant frequencies.

In addition to this, the relative fundamental frequency (RFF) was estimated from three consecutive tokens of /afa/, /ifi/, /ufu/ for each condition to estimate changes in laryngeal tension. The RFF analysis was carried out using a semi-automated analysis method adapted from Lien et al 2015[71] and was done using Octave ([GNU Octave] version 6.1.0, www.gnu.org/software/octave/index).[72] The custom-made Octave script detects the period boundaries for each utterance and suggests the most likely instances of vowel offset and onset which are visually confirmed by the investigator. In cases when incorrect instances of glottal onset and offset are automatically selected, the investigator can visually select the most appropriate options and correct the analysis. The RFF analysis focused on the offset cycle 10 ($offset_{10}$) and onset cycle one ($onset_1$) (refer to Steep et al 2010[73] for more details). The subject was asked to remain relaxed during the data collection and produce all utterances at his habitual pitch and loudness (only controlled by self-perceived effort due to auditory masking) with equal stress on the vowel surrounding the fricative. Auditory masking was done as per experiment one. EGG contact quotient (CQ) values were also analyzed.

Statistical analysis

Statistical analysis was performed using the R software (R: A language and environment for statistical computing, Vienna, Austria, www.r-project.org).[74] Data was compared across different conditions (time or segments) and between pre and post NEMS conditions using analysis of variance and *t* tests. Significance level was set to 5%, although comments on non-statistically significant results are offered when appropriate. Tukey's HSD post-hoc analysis and independent samples *t* test comparisons were also performed for multiple and pre-post conditions respectively.

RESULTS

Experiment 1 - *Assessment of laryngeal muscle activation across different frequencies of NMES using accelerometer and fiberoptic nasolaryngoscopy.*

In order to assess the impact of different frequencies of NMES on glottal configuration, the frequencies of 5,10,15,30,60 and 80 pulses per second at 5 mA were tested. Data was obtained during normal breathing with and without NMES determined by the VitalStim's rest-work time. Firstly, the root mean square (RMS) values from the accelerometer (ACC_{rms}) signals were used for the estimation of the movement of the neck surface due to muscle contraction at different NMES frequencies. Figure 3 shows ACC_{rms} values (multiplied by 1000) for different NMES frequencies. The largest ACC_{rms} of 1.17 units (dimensionless) was found at 10 pps and the minimum ACC_{rms} value of 0.36 units at 60 pps. Overall, the ACC_{rms} signal reduces as NMES frequency is increased.

To further assess changes in laryngeal muscle activation among different frequencies of NMES during normal breathing, glottal area waveform (GAW) signals were extracted from the fiberoptic nasolaryngoscopy recordings for two consecutive Vitalstim's rest-work time cycles using the GAT software's manual segmentation mode.

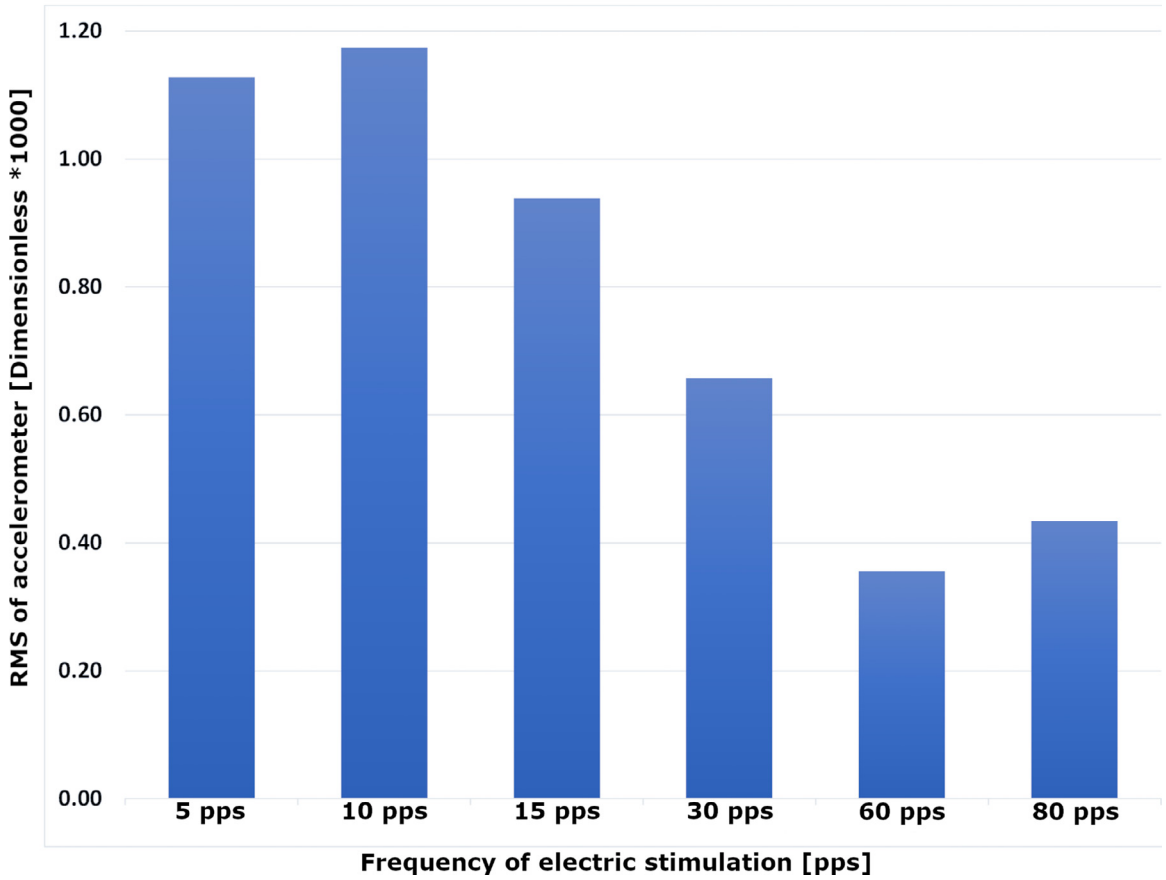

FIGURE 3. Estimation of movement of the neck surface at different NMES frequencies obtained as the RMS of the accelerometer signal. Data shows dimensionless values (Color version of the figure is available online.)

Maximum and minimum glottal area (GA) values for each NMES frequency were then identified for each GAW (Figure 4). Maximum values occurred during inspiration and minimum values during NMES. As data was collected only during normal breathing, GAW, and GA values represent dynamic and static levels of glottal adduction respectively. Consequently, GAW, and GA values decreased as glottal adduction increases. Increased glottal adduction during NMES can be seen for all frequencies of stimulation, with the least amount of adduction found for 60 pps and maximum amount of adduction for 5 and 10 pps. A Spearman's rank-order correlation was run to assess the relationship between minimum GA (analogous to maximum glottal adduction) and ACC_{rms} across NMES frequencies. A borderline negative significant correlation between the two variables was found (r_s (64) = -0.82, P= 0.05). The level of adduction can also be observed by the amount of approximation between arytenoids as well as the medial displacement of the ventricular folds. Furthermore, antero-posterior constriction due to arytenoids' tilt towards the glottic space can be observed for all frequencies of NMES. Although maximum and minimum GA values demonstrate the extreme changes in glottal adduction between normal breathing with and without NMES, they do not provide information regarding dynamic changes in GA within a NMES work-rest time cycle.

In order to assess this, the distribution of GA values (expressed as number of pixels) within each rest-work time cycle for all NMES frequencies are shown side-by-side in figure 5. Two successive rest-work time cycles were analyzed for each NMES frequency. In doing so, changes in GA were compared within and between NMES frequencies. The horizontal axis shows the frequencies of stimulation used in the analysis. The two tokens (ie, consecutive cycles) of rest-work time cycle recordings for each frequency can be identified by the subscripted number in the x axis. The vertical axis shows the GA values expressed as the number of pixels. The color bar on the right provides the value scale for GA occurrences. An 1860 bin size was used between the minimum (15876) and maximum (71883) GA values found for the entire dataset. Five and 10 pps were the only frequencies showing a bimodal distribution (peaks marked by the asterisks) which indicates that within a single rest-work time cycle, the GA presented two distinctive configurations (e.g., 5pps1 shows large incidences of glottal area with 21500 and 47500 number of pixels). Single prominent peaks with

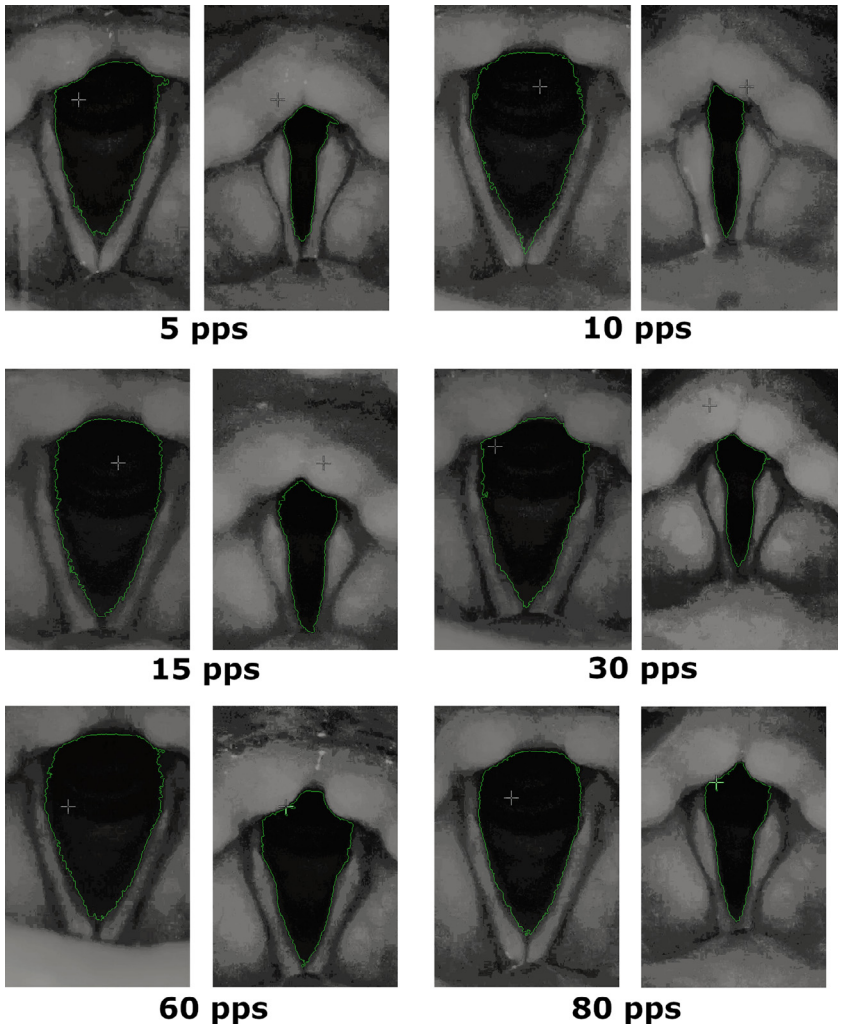

FIGURE 4. Glottal area comparison among different NMES frequencies. Paired (left and right) images show the larynx during normal inspiration (maximum GA value) and NMES (minimum GA value).

narrow distributions were found for 30pps1, 60pps1, and 60pps2. Large spreads of GA values were found for 5,10,15, and 30 pps and narrower ranges for 60 and 80 pps. The smallest values for the GA were found for frequencies between 5 and 30 pps. The second token for 30 pps showed a similar distribution to 60 pps.

Experiment 2 - *Assessment of changes in laryngeal configuration using traditional and high-speed videoendoscopy during a 12-minute long NMES session.*

In order to assess cumulative changes in laryngeal configuration during NMES, a 12-minute-long session was carried out whilst both traditional laryngeal (10fps) and HSV (6000fps) video recordings were obtained. Traditional video was used to record the entire session and assess changes in laryngeal configuration during normal breathing, whilst HSV was used during phonation.

Although lower frequencies produced larger changes in glottal configuration in experiment one, the NMES device was set to 35 pps as it produced comfortable and smooth muscle activation. This choice is supported by the fact that lower frequencies produced perceivable muscle twitches during experiment one which could lead to possible unconscious laryngeal adjustments during data collection. Figure 6 shows the laryngeal configuration during normal breathing at rest and during NMES at 35 pps. Changes in glottal and supraglottal configuration similar to experiment one were observed.

Likewise, in experiment one, GA values during normal breathing (here, also analogous to glottal adduction levels) were extracted from the traditional laryngeal video recording using the GAT software. However due to natural physiological behaviors such as swallowing and the movement of the epiglottis, only a subpart of the first three minutes of NMES showed the view of the glottis without obstructions. Beyond the first three minutes of NMES, the epiglottis constantly obstructed the view of the larynx, therefore hindering image analysis. The horizontal bar (top) in figure 7

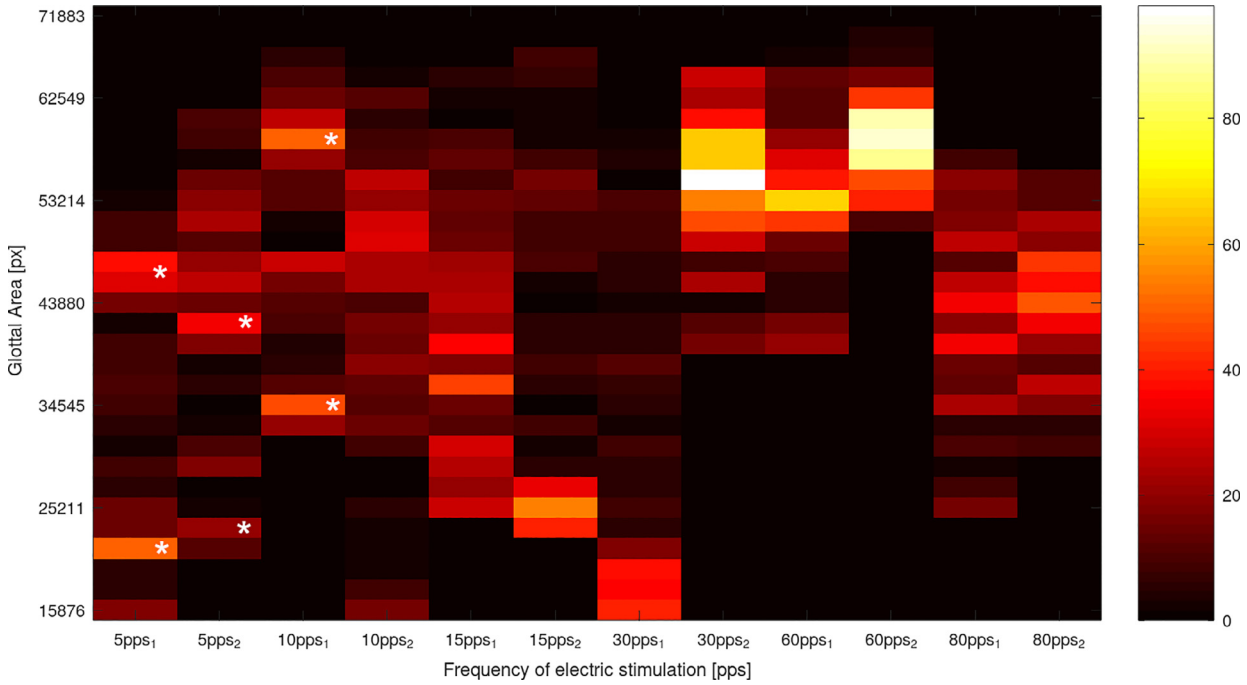

FIGURE 5. Distribution of number of pixels (extracted from the glottal area waveform) across different electric stimulated frequencies. The subscripted numbers accompanying the frequency values refers to the token number. The color bar on the right shows the occurrence of frames (used for the glottal area extraction) with each specific glottal area (no. of pixels). Bin size was set to 1860 pixels. The asterisks indicate the peaks of the bimodal distributions (Color version of the figure is available online.)

shows the ratio between accepted (blue) and rejected (red) frames. Accepted frames were extracted and concatenated. Figure 7 (middle) shows the GA for the concatenated frames plotted across time. Five concatenated *GA segments* are shown with vertical red lines marking the interruptions in the recording where the glottis was not fully visible. The length of each break interval is annotated beside the lower part of the red lines. The breathing pattern can be estimated by the peaks in the GA when inspiratory forces abduct the glottis. Prolonged local minimas in the GA can be seen beyond 40 seconds and correlate to NMES work-time, also estimated from the ACC signal (bottom graph). A small delay between the onset of the ACC signal and the decrease in GA is seen between signals for each rest-work time cycle. The lowest GA values within each rest-work time cycle were found during or just after NMES. Larger extreme values for the GA are shown in the first segment when compared to later ones. A similar pattern is also observed in the ACC signal. In addition to this, changes in GA during inspiration (ie, peaks in the GA signal) and the overall mean GA decreased across NMES.

In addition to changes in GA during NMES, changes in laryngeal configuration were also analyzed using the accepted frames. In order to avoid instances where GA values (and likely laryngeal configuration) were influenced by inspiration or NMES, mean values for each GA segment were calculated (Figure 7 middle, horizontal dashed lines). Despite the GA segments not being evenly distributed in time or having the same duration, the changes in mean GA values between segments demonstrate the effect of NMES on GA. The mean values were used as a reference for the identification of frames with laryngeal configuration closer to neutral (see figure 8 for an example). Of these, five nonconsecutive frames from each segment were selected for the laryngeal configuration analysis (Figure 7 middle, black circles).

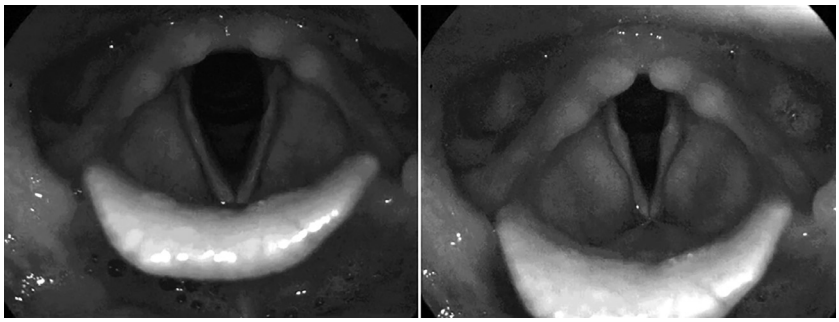

FIGURE 6. Laryngeal view at rest time (left) and during NMES stimulation at 35 pps (right).

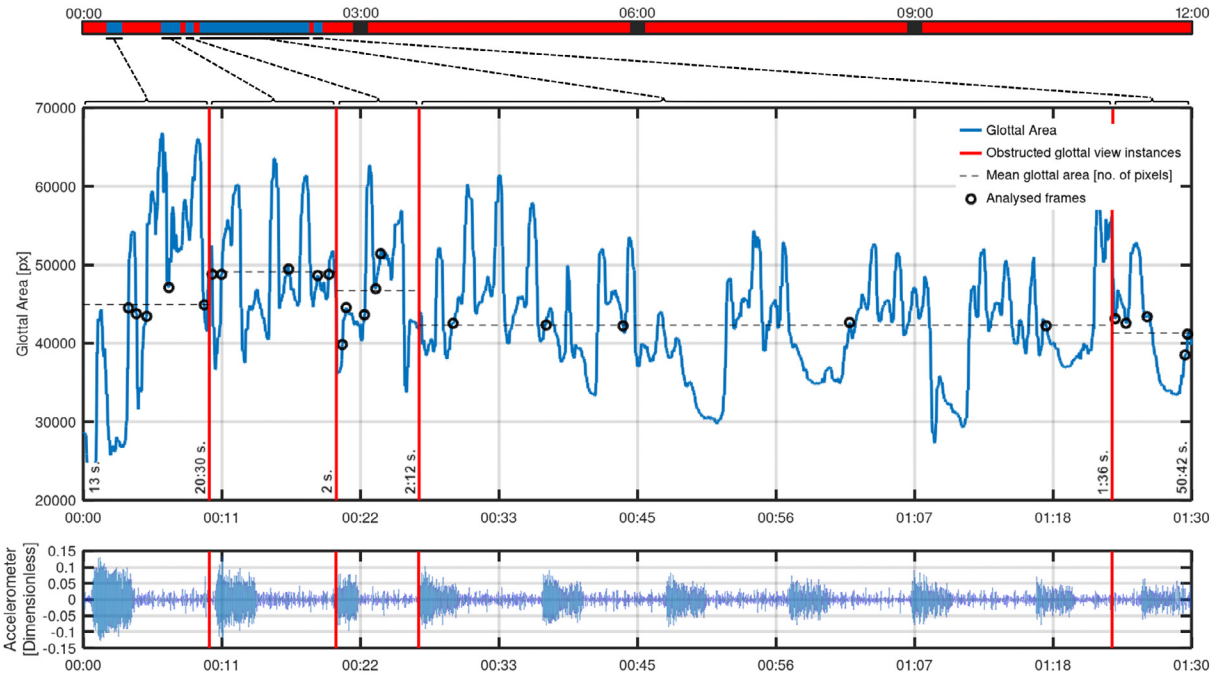

FIGURE 7. The horizontal bar (top) visually displays the analyzed (blue) and rejected (red) frames. Dark gray segments represent HSV recordings. The middle and bottom figures show GA and ACC signals respectively for the initial 1:30 non-consecutive seconds of NMES stimulation during normal breathing. Vertical red lines show interruptions in the recording due to unwanted movement, swallowing or steaming of the lenses. The duration of each interruption is annotated beside each red line marking the length of interruptions. The horizontal dashed lines represent the mean GA values for each segment while the black circles indicate the frames used for the analysis of changes in laryngeal configuration across time (Color version of the figure is available online.)

The laryngeal measurements were: Interarytenoid space, posterior glottal width, distance between aryepiglottic lateral angles, antero-posterior glottal width, vocal fold and false vocal fold angles (Figure 8). Laryngeal measurements were made using the ImageJ software. In order to compare values among images, all measurements were calculated as a percentage of the width of the epiglottis which was used as reference. Measurements of angles did not require a reference value.

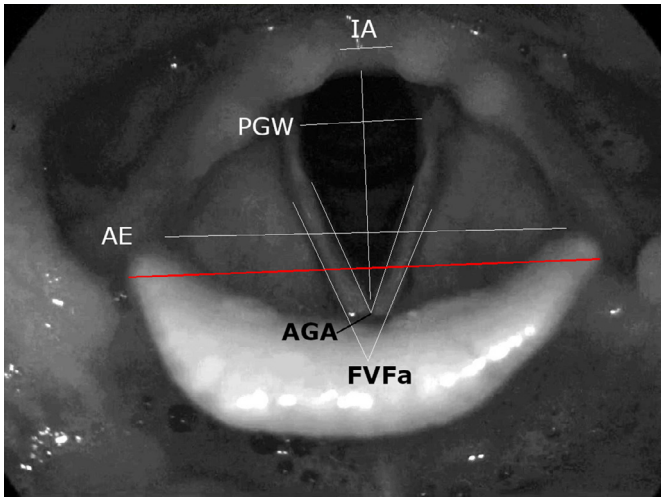

FIGURE 8. Shows the measurements used for the analysis of the glottal and supraglottal configuration. AE, distance between aryepiglottic angles; APG, antero-posterior glottal width; FVFa, anterior false vocal fold angle IA, Inter-arytenoid distance; PGW, posterior glottal width and VFa, anterior vocal fold angle. The width of the epiglottis was used as reference for the analyses (red) (Color version of the figure is available online.)

Changes in laryngeal configuration are summarized in table 1. No significant changes were found for interarytenoid space and posterior glottal width. The distance between aryepiglottic lateral angles was significantly different between segment three and, one, four and five showing a narrower lateral constriction in segment three. Antero-posterior glottal width values were significantly different between section one and five with progressive narrowing of the glottis with NMES. A significant reduction in the anterior angle of the glottis was found between segment one when compared to the other segments. A similar result was found for the anterior angle of the false vocal folds with a significant difference between segments one and two.

HSV recordings at 6000fps during phonation were also performed every three minutes, and at the beginning and the end of NMES (ie, 0,3,6,9 and 12 minutes). HSV recordings within the NMES session are shown as dark gray segments in figure 7 (top). Changes in glottal area (open quotient, closing quotient, glottis gap index, and glottal area index) and mechanical measures (stiffness, peak closing velocity, peak acceleration, and amplitude length ratio) were analyzed using the GAT software.

The results from the HSV analysis are shown in table 2. All variables showed statistically significant changes across

TABLE 1.
Statistical Data for Laryngeal Configuration Analysis

	Mean (SD) (Calculated as Percentages of the Epiglottis Width)						
Laryngeal Measurements	Segment 1	Segment 2	Segment 3	Segment 4	Segment 5	ANOVA F-Value (*P*-value)	Tukey HSD Post-Hoc Comparison (Significant Difference Between Groups)
Interarytenoid space	0.08(0.015)	0.08(0.018)	0.09(0.016)	0.08(0.004)	0.08(0.008)	0.49(0.74)	na
Posterior glottal width	0.22(0.021)	0.23(0.011)	0.232(0.015)	0.22(0.00)	0.22(0.01)	0.67(0.62)	na
Distance between arye-piglottic lateral angles	0.83(0.018)	0.8(0.017)	0.79(0.022)	0.84(0.019)	0.83(0.024)	6.03(0.00)†	3-1,4,5
Antero-posterior glottal width	0.47(0.01)	0.46(0.01)	0.46(0.00)	0.45(0.00)	0.44(0.00)	4.25(0.01)‡	1-5
Anterior glottal angle	43.02(4.22)	35.09(1.29)	34.99(2.08)	32.48(0.62)	32.82(2.94)	13.9(0.000)*	1-2,3,4,5
Anterior false vocal folds angle	41.95(1.74)	38.59(1.45)	39.95(2.43)	39.4(1.53)	41.03(1.04)	3.04(0.041)‡	1-2

* 0.001
† 0.01
‡ 0.05
na, non-applicable
Significance codes:

the five assessment points as well as between pre and post NMES intervention. Apart from the *closing quotient*, changes were unidirectionally progressive with NMES for all glottal area measures. *Open quotient* and *Glottis Gap Index* decreased whilst *glottal area index* increased. No clear trend was observed for the closing quotient. Changes in mechanical measures were also significant for all parameters. An overall increase was found for *stiffness* whilst *n*o clear trends were found for the other variables. *Peak Closing Velocity* and *Peak Acceleration* decreased between the pre- and post-conditions whilst *Amplitude Length Ratio* increased. Large effect sizes were found for all variables using Cohen's d statistical test.

Experiment 3 - *Acoustic analysis during a 12-minute long NMES session.*

In order to exclude possible influences of head position on voice quality due to the endoscopic procedure, a third experiment devoted to the acoustic analysis of the voice was carried out. Similar to experiment two, a 12-minute long NMES session using 35 pps with five points of assessment (t = 0,3,6,9, and 12 minutes) was carried out. F_0, formant analysis, SPL, CPPS, LHSR and CQ results are shown in table 3. Due to a technical issue, the 2nd acoustic measurement (t_3) for vowel /u/ was not correctly recorded and was excluded from the analysis.

The results for the acoustic analysis showed a significant increase in F_0 with NMES for all vowels. A significant statistical difference was also found for the same variable between the before (t_0) and after (t_{12}) NMES conditions using an independent samples t test. The formant frequency analysis showed a significant change for formants three and four with inconsistent directions among vowels. Changes in SPL were also statistically significant showing higher values after NMES. SPL comparison for pre-post NMES was also significant for all vowels. Significant increase in CPPS values were found for vowel /i/ and /u/. No significant changes were found for the vowel /a/ which showed an opposite trend when compared to the other vowels where values decreased with NMES. Changes in LHSR values were significant for /i/ and /u/ however no clear trends were observed across the five points of assessment. Changes in contact quotient (CQ) showed overall significant changes for all vowels however no clear trends were observed. Significant increase in CQ and CPPS values were found between the pre-post NMES condition.

In order to investigate the possibility of NMES causing increased tension on the laryngeal muscles, a relative

TABLE 2.
Statistical Data for Glottal Area and Mechanical Measures Using the Glottis Analysis Tools Software

	GAT Analysis (unit)	Mean and Standard Deviation Values across 5 Assessment Points (t_0 to t_{12})	ANOVA (*P*-value) | TukeyHSD Post Hoc	t_0 - t_{12} Independent Samples *t* test (*P*-value) |Cohen's d
Glottal area measures	Open Quotient (Dimensionless)	t_0 = 0.9996 (0.003) t_3 = 0.9996 (0.003) t_6 = 0.9991 (0.005) t_9 = 0.96 (0.03) t_{12} = 0.86 (0.05)	423.2 (0.000*)All, apart from t_0-$t_{3,6}$; t_3-$t_{0,6}$; t_6-$t_{0,3}$	25.13 (0.000*) | 3.65
	Closing Quotient (Dimensionless)	t_0 = 0.54 (0.02) t_3 = 0.53 (0.02) t_6 = 0.59 (0.01) t_9 = 0.57 (0.02) t_{12} = 0.46 (0.05)‡	233.4 (0.000*)All, apart from t_0-t_3	12.84 (0.000*) | 1.86
	Glottis Gap Index (Dimensionless)	t_0 = 0.017 (0.002) t_3 = 0.012 (0.003) t_6 = 0.015 (0.001) t_9 = 0.001 (0.002) t_{12} =0.0000 (0.000)	1386 (0.000*)All	78.17 (0.000*) | 11.02
	Glottal Area index (Dimensionless)	t_0 = 0.981 (0.005) t_3 = 0.989 (0.003) t_6 = 0.987 (0.006) t_9 = 1.031 (0.03) t_{12} = 1.174 (0.06)	462.2 (0.000*)All, apart from t_0-$t_{3,6}$ and t_3-t_6	25.47 (0.000*) | 3.7
Mechanical measures	Stiffness(1/s)	t_0 = 936.46 (41.96) t_3 = 954.14 (82.88) t_6 = 912.01 (46.48) t_9 = 1147.88 (110.01) t_{12} = 1122.13 (75.02)	219 (0.000*)All, apart from t_0-$t_{3,6}$ and t_9-t_{12}	21.23 (0.000*) | 3.06
	Peak Closing Velocity (Mpx/s)	t_0 = 1.29 (0.036) t_3 = 1.46 (0.044) t_6 = 2.09 (0.056) t_9 = 1.07 (0.037) t_{12} = 0.95 (0.042)	10430 (0.000*)All	59.54 (0.000*) | 8.54
	Peak Acceleration (Mpx/s^2)	t_0 = 1633.23 (77.29) t_3 = 1772.78 (90.35) t_6 = 2805.08 (142.49) t_9 = 1399.91 (86.49) t_{12} = 1164.47 (79.73)	4176 (0.000†)All	41.66 (0.000*) | 5.97
	Amplitude Length Ratio (Dimensionless)	t_0 = 16.74 (0.21) t_3 = 19.66 (0.98) t_6 =21.23 (0.31) t_9 = 15.30 (0.62) t_{12} = 17.24 (0.32)	1833 (0.000*)All	12.49 (0.000*) | 1.8

* 0.001.
† 0.01.
‡ 0.05.
Significance codes:

fundamental frequency (RFF) analysis was performed for three consecutive tokens of /afa/, /ifi/ and /ufu/ across the five assessment points. $Offset_{10}$ and $onset_1$ were calculated for each vowel separately.

Statistical summary of the RFF analysis is shown in table 4. Overall RFF values for $offset_{10}$ and $onset_1$ reduced with NMES, suggesting an increase in laryngeal tension. Significant values were found for /ifi/ $onset_1$ and /ufu/ $onset_1$ and $offset_{10}$. In order to ascertain pre-post treatment effect, independent samples *t* test comparison between t_0 and t_{12} were also calculated. The results showed statistically significant differences for /ifi/ $onset_1$ and /ufu/ $offset_{10}$.

TABLE 3.
Acoustic Analysis Results. Correction Factor of 2.2dB CPPS/10 dB SPL Difference Applied According to Brockmann-Bauser et al (2019)

	Vowels					
	A		i		u	
Acoustic Parameters	Mean (SD)	ANOVA (*P*-Value) | TukeyHSD Post Hoc | t_0 - t_{12} Independent Samples *t* test (*P*-value)	Mean (SD)	ANOVA (*P*-value) | TukeyHSD Post Hoc | t_0 - t_{12} Independent Samples *t* -test (*P*-value)	Mean (SD)	ANOVA (*P*-Value) | TukeyHSD Post Hoc | t_0 - t_{12} Independent Samples *t* -test (*P*-value)
F_0	t_0 = 136.74 (0.15) t_3 = 162.48 (1.25) t_6 =161.46 (5.73) t_9 =163.19 (0.55) t_{12} =163.64 (3.05)	45.97 (0.000)* | t_0-$t_{3,6,9,12}$ | -15.23 (0.004)†	t_0 = 138.19 (0.75) t_3 = 169.03 (5.82) t_6 =174.99(1.37) t_9 =172.05 (1.82) t_{12}= 170.60 (3.65)	64.74 (0.000)* | t_0-$t_{3,6,9,12}$ | -15.03 (0.003)†	t_0 = 140.61 (0.31) t_6 = 173.48 (1.74) t_9 = 178.32 (0.97) t_{12} = 177.44 (1.55)	599.5 (0.000)* | t_0-$t_{6,9,12}$; t_6-$t_{9,12}$ | -40.23 (0.000)*
F_1	t_0 = 704.86 (15.30) t_3 = 666.21 (30.29) t_6= 743.19 (28.33) t_9 = 703.37 (35.54) t_{12} = 710.87 (23.42)	2.98 (0.07)§ | na | -0.37 (-0.73)	t_0 = 390.14 (121.08) t_3 = 338.77 (12.03) t_6 = 351.99(4.47) t_9 = 345.23 (3.25) t_{12} = 342.75 (7.46)	0.43 (-0.77) | na | 0.67 (-0.56)	t_0 = 393.45 (22.7) t_6 = 349.1 (3.67) t_9 = 361.77 (3.14) t_{12} = 359.52 (2.42)	8.07 (0.008)† | t_0-$t_{6,9,12}$ | 2.57 (-0.12)
F_2	t_0 = 1308 (34.39) t_3 = 1301.81 (38.80) t_6=1307 (38.46) t_9 = 1252.82 (154.68) t_{12} = 1364.68 (55.56)	0.75 (-0.57) | na | -1.49 (-0.22)	t_0 = 2311.57 (122.20) t_3 = 2261.14 (14.46) t_6 = 2272.21(5.74) t_9 = 2286.66 (3.40) t_{12} = 2235.16 (21.93)	0.77 (-0.56) | na | 1.06 (-0.39)	t_0 = 1152.31(240.18) t_6= 982.56 (34.48) t_9 = 1068.19 (7.58) t_{12} = 1058.34 (5.44)	0.98 (-0.44) | na | 0.67 (-0.56)
F_3	t_0 = 2391.75 (33.77) t_3 = 2244.23(98.46) t_6 = 2477.57 (17.76) t_9 = 2345.94 (142.83) t_{12} = 2437.45 (48.78)	3.59 (0.04)‡ | t_3-t_6 | -1.33 (-0.26)	t_0 = 2760.2 (75.58) t_3 = 2630.57(40.07) t_6 = 2663.84(13.28) t_9 = 2629.76 (42.53) t_{12} = 2558.36 (33.37)	7.74 (0.004)† | t_0-$t_{6,9,12}$ | 4.23 (0.02)‡	t_0 = 2707.37 (100.51) t_6 = 2533.49 (46.13) t_9 = 2493.58 (79.21) t_{12} = 2474.7 (66.74)	5.9 (0.02)‡ | t_0-$t_{6,9,12}$ | 3.33 (0.03)‡
F_4	t_0 = 3872.04 (28.65) t_3 = 3875.04(48.99) t_6 = 3940.89 (492.25) t_9 = 3868.28 (44.78)	1.01 (-0.44) | na | -9.57 (0.002)†	t_0 = 4015.4 (19.35) t_3 = 4156.78(116.54) t_6 = 4109.47(29.16) t_9 = 4098.75 (25.21)	2.52 (-0.1) | na | -6.04 (0.003)†	t_0 = 3881.09 (84.34) t_6 = 3805.53 (53.83) t_9 = 3924.50 (84.49) t_{12} = 3904.99 (43.00)	1.71 (-0.24) | na | -0.43 (-0.69)

(*Continued*)

TABLE 3. (*Continued*)

	Vowels					
	A		i		u	
Acoustic Parameters	Mean (SD)	ANOVA (*P*-Value) | TukeyHSD Post Hoc | t_0 - t_{12} Independent Samples *t* test (*P*-value)	Mean (SD)	ANOVA (*P*-value) | TukeyHSD Post Hoc | t_0 - t_{12} Independent Samples *t* -test (*P*-value)	Mean (SD)	ANOVA (*P*-Value) | TukeyHSD Post Hoc | t_0 - t_{12} Independent Samples *t* -test (*P*-value)
t_{12} = 4154.59 (51.88)	t_{12} = 4114.69 (20.86)					
SPL@30 $cm_{Relative}$	t_0 = 60.76 (0.26) t_3 = 70.08 (1.43) t_6 = 71.61 (0.62) t_9 = 68.98 (1.66) t_{12} = 69.81 (1.22)	40.67 (0.000)* | t_0-$t_{3,6,9,12}$ | -12.47 (0.004)†	t_0 = 55.61 (4.84) t_3 = 68.55 (0.20) t_6 = 70.07 (0.42) t_9 = 69.19 (0.94) t_{12} = 68.70 (1.61)	20.35 (0.000)* | t_0-$t_{3,6,9,12}$ | -4.43 (0.03)‡	t_0 = 60.95 (0.66) t_6 = 71.9 (0.27) t_9 = 72.14 (0.42) t_{12} = 71.16 (0.62)	321 (0.000)* | t_0-$t_{6,9,12}$ | -19.31 (0.000)*
CPPs	t_0 = 17.29 (0.34) t_3 = 15.43 (0.6) t_6 = 16.68 (1.37) t_9 = 15.86 (0.24) t_{12} = 16.07 (0.33)	3.11 (0.06)§ | na | 4.37 (0.01)‡	t_0 = 14.34 (1.38) t_3 = 13.44 (0.81) t_6 = 14.35 (0.75) t_9 = 13.26 (0.29) t_{12} = 15.1 (0.40)	2.48 (-0.11) | na | -0.9 (-0.44)	t_0 = 12.93 (0.20) t_6 = 12.24 (0.13) t_9 = 13.16 (0.22) t_{12} = 13.64 (0.60)	8.39 (0.007)† | t_6 - $t_{9,12}$ | -1.9 (0.17)†
LHSR	t_0 = -35.28 (0.27) t_3 = -33.92 (0.83) t_6 = -35.75 (1.26) t_9 = - 36.33 (0.41) t_{12} = -35.56 (1.29)	2.86 (0.08)§ | na | -12.47 (-0.74)	t_0 = -26.39 (0.27) t_3 = -31.39 (2.72) t_6 = -25.62 (0.35) t_9 = -28.49 (0.29) t_{12} = -26.26 (0.28)	10.72 (0.001)† | t_0-t_{12}; t_3-$t_{6,12}$ | -0.54 (-0.61)	t_0 = -34.29 (2.5) t_6 = -46.93 (0.31) t_9 = -45.73 (2.20) t_{12} = -45.5 (0.87)	35.01 (0.000)* | t_0-$t_{6,9,12}$ | 7.32 (0.009)†
CQ	t_0 = 0.49 (0.01) t_3 = 0.53 (0.01) t_6 = 0.53 (0.00) t_9 = 0.49 (0.02) t_{12} = 0.50 (0.01)	5.61 (0.01)‡ | t_0-$t_{3,6}$ | -1.43 (-0.22)	t_0 = 0.47 (0.01) t_3 = 0.51 (0.02) t_6 = 0.49 (0.00) t_9 = 0.47 (0.00) t_{12} = 0.5 (0.00)	4.77 (0.02)‡ | t_0-t_3 | -4.75 (0.04)‡	t_0 = 0.49 (0.00) t_6 = 0.50 (0.01) t_9 = 0.51 (0.01) t_{12} = 0.53 (0.01)	3.48 (0.07)§ | na | -3.42 (0.04)‡

* 0.001
† 0.01
‡ 0.05
§ 0.1
Significance codes:

TABLE 4.
RFF Statistical Analysis

	Offset10				Onset1			
RFF Task	Mean (SD)	ANOVA F value (*P*-value)	Tukey HSD Post-hoc Group Differences	Independent Samples *t* test Comparison (t_0 vs t_{12}) (*P*-value)	Mean (SD)	ANOVA F Value (*P*-value)	Tukey HSD Post-hoc Group Differences	Independent Samples *t* test Comparison (t_0 vs t_{12}) (*P*-value)
afa	t_0= -0.13 (0.34) t_3= -1.07 (0.48) t_6= -0.59 (0.68) t_9= -1.21 (0.05) t_{12}= -1.14 (0.65)	2.16 (0.15)	na	2.37 (0.09)	t_0 = 2.81 (1.32) t_3= 2.11 (1.6) t_6= 3.01 (0.8) t_9= 0.93 (0.65) t_{12}= 1.97 (0.88)	1.21 (0.37)	na	0.91 (0.42)
ifi	t_0 = -0.8 (0.71)* t_3= -1.57 (0.62) t_6= -0.84 (1.02) t_9= -2.16 (0.67) t_{12}= -2.38 (0.82)	2.51 (0.11)	na	2.51 (0.06)	t_0 = 2.14 (0.66) t_3= 0.63 (0.88) t_6= 0.23 (0.44) t_9= 0.11 (0.44) t_{12}= -0.24 (0.44)	6.84 (0.006†)	1-3,4,5	5.19 (0.009†)
ufu	t_0 = -0.54 (0.76) t_3= -2.84 (0.81) t_6= -2.61 (0.39) t_9= -1.8 (0.12) t_{12}= -2.46 (0.13)	4.36 (0.002†)	1-2.3.5	4.26 (0.04‡)	t_0 = 1.72 (1.4) t_3= 1.46 (1.7) t_6= -0.35 (1.01) t_9= -1.09 (1.02) t_{12}= -0.13 (0.46)	3.53 (0.04‡)	1-4	2.15 (0.14)

* 0.001.
† 0.01.
‡ 0.05.
Significance codes:

DISCUSSION

Experiment 1 - *Assessment of laryngeal muscle activation across different frequencies of NMES using accelerometer and fiberoptic nasolaryngoscopy.*

Changes in laryngeal muscle activation across different NMES frequencies was firstly assessed using the RMS of the accelerometer signal (ACC_{rms}). The ACC_{rms} was used to estimate the movement of the neck surface due to muscle contraction during different frequencies of NMES. A clear trend was found where the ACC_{rms} decreased as NMES frequency increased. The higher ACC_{rms} values found for the lower frequencies could have been caused by muscle twitches which produce larger changes in acceleration, whilst greater stimuli summation may have been present at higher frequencies showing lower ACC_{rms} values. This difference in muscle activation was also perceived by the tested subject where a pulsating contraction for five and 10 pps was subsequently replaced by a smooth tightening of the laryngeal muscles for higher frequencies. Furthermore, the ACC_{rms} also showed a negative correlation with the minimum glottal area values presented in figure 4. As during normal breathing glottal area is inversely proportional to glottal adduction, this result means that the movement on the neck surface and glottal adduction decreased as NMES frequency increased. Unexpectedly, this result is in disagreement with previous findings from NMES applied to body limbs[31,32] that increasing frequency of NMES produces stronger muscle activation. And although in this study a clear trend between glottal adduction and NMES frequency was found, this relationship may not be so clear with other methods of ES. While transcutaneously stimulating the recurrent laryngeal nerve of monkeys, Sanders et al 1987[75] found that glottal adduction did not systematically change with changes in frequency. According to him frequencies between 10-30 Hz generated maximum glottal abduction whilst stimulation above 30 Hz caused glottal adduction with complete glottal closure at 100 Hz. His results are not directly comparable to this study as different methods of stimulation, species, and targeted structures were used; however, it does support the notion that different frequencies of stimulation activate structures in different ways. We aim to further investigate the influences of NMES frequency on laryngeal muscle activation as the results from this study challenge the common use of 80 pps for voice patients, especially if treatment is aimed at laryngeal adduction.

The visual inspection of the larynx using fiberoptic nasolaryngoscopy reveals that NMES stimulation causes changes in laryngeal configuration by increasing adduction, interarytenoid, and antero-posterior approximation, and lateral medialization of the ventricular folds. These changes confirm the ability of NMES (as used in this study) to penetrate beyond the external structures of the larynx to activate the intrinsic laryngeal muscles. Considering the alternative electrode placement used in this study, it is likely that the CT muscles were activated. However, their activation cannot be confirmed in experiment one as it is not easily identifiable from the traditional endoscopic viewing plane. However, the activation of other intrinsic muscles, such as the lateral cricoarytenoid (LCA) and interarytenoid (IA) muscles are evidenced by the increased adduction levels during NMES. It is also unclear from experiment one whether NMES activates the thyroarytenoid (TA) and posterior cricoarytenoid muscles. Nevertheless, the unusual concave shape at the posterior region of the glottis may be the cause of the co-activation of the LCA and TA muscle as it seems to mark the transition between the membranous and cartilaginous parts of the vocal folds. A similar concave shape is seen in the anterior part of the ventricular folds which, in addition to the antero-posterior and lateral approximation of structures above the glottis, indicated that the NMES also influenced supraglottic structures.

Glottal area (here analogous to glottal adduction, henceforth referred to as glottal adduction) also varied across rest-work time cycles for different NMES frequencies. Changes in glottal adduction within a single rest-work time cycle were not homogeneous between or within NMES frequencies. Glottal adduction for five and 10 pps presented a bimodal distribution within a single rest-work time cycle showing a clearer impact of NMES on glottal adduction when compared to higher frequencies. Overall, lower NMES frequencies produce stronger intrinsic laryngeal muscle activation demonstrated by larger neck movement (ACC_{rms}), greater glottal adduction during maximum NMES and more prominent changes in glottal adduction values across rest-work time cycles.

Experiment 2 - *Assessment of changes in laryngeal configuration using traditional and high-speed video endoscopy during a 12-minute long NMES session.*

In relation to cumulative changes in laryngeal configuration, the results from experiment two showed a progressive increment in glottal adduction across the 12 minutes of NMES. In addition to this, the impact of breathing on GA values became less pronounced as glottal adduction levels increased. It is likely that these changes are related to muscle stimuli summation leading to increased tension in the larynx. Contrary to previous studies suggesting unlikely intrinsic muscle activation with NMES,[36–38] our results showed clear changes in glottal adduction in the first few minutes of application. The large effect found in our study is attributed to changes in electrodes' size and placement and the use of a lower frequency of NMES (35 pps) when compared to previous studies. The cumulative effects of NMES are further observed by the activation of supraglottic structures evidenced by the inability to visually observe the entire glottis beyond merely three minutes of NMES.

Still in experiment two, the assessment of changes in laryngeal configuration further evidences the activation of different laryngeal muscles across time. As this analysis

(Table 1) was done on video frames in which the larynx was least influenced by inspiration or NMES (ie, at rest), it more accurately represents progressive effects of NMES on laryngeal configuration over time. Changes in laryngeal configuration are caused by the activation of intrinsic laryngeal muscles. In specific, CT activation was inferred from changes in the anterior angle of the glottis by Humbert et al 2008.[38] In our study, a 23.7% significant reduction of the anterior angle of the glottis between before and just after a few minutes of intervention, demonstrates that NMES can also activate the CT muscles producing significant changes to the glottis. Although not directly comparable, as different electrode sizes and placements, frequency of stimulation, and client groups were assessed, this result corroborates the ability of activating the CT muscle using NMES found by Humbert et al 2008.[38] Nevertheless, their conclusion was that changes in CT activation were not large enough to grant clinical validity. This is not shared by our findings, as the observed significant changes found in this study may well suit clinical therapy for voice patients. Significant changes at the beginning of the session were also found for the anterior angle of the false vocal folds. It is likely that these changes became more pronounced after the entire session which lasted 12 minutes. However, due to viewing obstruction of the larynx by the petioles and tip of the epiglottis, further changes were not assessed. The significant narrowing of the antero-posterior glottal width was probably caused by the combined activation of the LCA and TA muscles in addition to other supraglottic structures. The PCA muscles were likely not activated to the same degree as other intrinsic muscles as no significant changes in the posterior glottal width were observed, therefore suggesting that NMES was weaker or absent in the posterior part of the glottis.

In the final analysis of experiment two, HSV assessment of voice production at five equally spaced intervals (0,3,6,9 and 12 minutes) using the GAT software shows significant changes in vocal fold vibration. Glottal area measures showed a decrease in the range of glottal area values (decreased *glottal gap index*) with time. Furthermore, the glottis progressively remained closed for longer (decreased open quotient) with faster changes between opened and closed (higher glottal area index), in specific during closing of the glottis (decreased closing quotient) with NMES. These results showed increasing levels of laryngeal muscles activation with NMES, where faster changes of a smaller glottal area take place with longer closed phases within a cycle. Gradual changes were observed for all variables apart from the closing quotient. In addition to this, changes in mechanical measures showed reduced values for peak closing velocity and acceleration with higher values for stiffness when compared between the time intervals of 0 and 12. The apparently conflicting results between GAT glottal area and mechanical measures that show faster changes between open and closed glottis, albeit with lower peak closing velocity and acceleration, are likely due to the overall more adducted glottis found for NMES. Changes in stiffness further confirm the ability of intrinsic laryngeal muscle activation using NMES. Overall, NMES was able to cause changes in glottal adduction and configuration within less than three minutes of activation. The changes were progressive towards hyperfunction.

Experiment 3 - *Acoustic analysis during a 12-minute long NMES session.*

The acoustic analysis in experiment three further supports the findings from the image analysis, that NMES causes significant changes in the vocal apparatus via the activation of intrinsic laryngeal muscles. In specific, a 20% increase in F_0 further evidences a progressive CT muscle activation with NMES.

Sundberg and Nordstrom 1976[76] found that changes in laryngeal height, in specific laryngeal elevation, was associated with increased F_3 and F_4 in the acoustic analysis. In experiment three, changes in formant frequencies were observed for F_3 and F_4, however as they were in opposite directions and varied across vowels, changes in vertical laryngeal position as a consequence of suprahyoid muscle activation were not evidenced with the method of NMES used in this study. Changes in voice output were reflected by the larger SPL values found after NMES. The increased SPL values from t_0 to t_{12} could have been influenced by larger subglottal pressures caused by the increased laryngeal resistance. However, as during the experiment the subject remained relaxed and phonated at a habitual level, the SPL gain was more likely caused by the increased glottal closure instead of subglottal pressure alone. Changes in voice spectrum as measured by the CPPS and LHSR were inconclusive and showed somewhat opposite trends for /a/ and /i/ when compared to /u/ vowels. Overall, CQ values increased with NMES which was previously observed by the changes in open quotient in the HSV analysis. In addition to this, the overall RFF values for all vowels decreased with NMES indicating a higher level of tension in the laryngeal muscles. The RFF results showed significant changes for vowels /i/ and /u/ but not for /a/. This result agrees with Park and Stepp 2019[77] that found closed vowels to be more sensitive to RFF changes than open vowels, in specific for onset.[1] As increased values for F_0, SPL and CQ, as well as lower RFF values were found, the acoustic analysis (experiment 3) further evidences the higher level of muscle activation and laryngeal tension after NMES found in experiments one and two.

Considering the results of all three experiments, it appears that NMES may be a good candidate to counteract the effects of glottal insufficiency, as it is able to produce large activation of the intrinsic laryngeal muscles. However, in order to achieve the desired results in a safe way, it is important to consider all NMES parameters used for the treatment of voice patients. Compared to previous studies, our use of smaller electrodes placed along the length of the lower margin of the thyroid cartilage was effective in

activating most adductor muscles of the larynx. Perhaps, a vertical placement of the electrodes along the horns of the thyroid cartilage may produce different results with activation of the abductor muscles. We aim to investigate alternative electrode placements in our future studies. Changes in NMES frequency showed to produce different levels of laryngeal muscle activation, with lower frequencies producing more extreme changes in glottal adduction. Consequently, lower NMES frequencies should be used to promote glottal adduction. The VitalStim guideline advises the use of one-hour long sessions for patients with swallowing disorders, however, our method of stimulation produced significant results within the first three minutes of electrical stimulation (with cumulative effects). Therefore, care should be taken to avoid muscle damage when using prolonged NMES sessions, especially when small electrodes that produce a more concentrated electric current are used. Additionally, muscle activation with electric stimulation differs from natural *in vivo* muscle activation, as it engages all muscle fibers at once and sustains longer periods of activation. Also, it is likely that the small laryngeal muscles may be more prone to fatigue when high intensities of NMES are used. Despite large levels of muscle activation were achieved in the three experiments, no symptoms of delayed-onset muscle soreness were experienced by the subject.

The single subject design in this pilot study poses a challenge to the generalization of some of the results. For example, it is unclear whether different NMES frequencies would produce the same results for other individuals. However, it is important to consider the multiple data collection sessions carried out, one week apart, as well as the different methods of analyses used in this study. Yet all the results show an agreement with regards to changes in glottal configuration, voice parameters and stiffness. The complex methodology, in specific the acquisition of image data where the tested subject needs to stay still for the length of the NMES, with the endoscope in situ at a stable distance from the glottis, poses a challenge to recruitment and should be considered when designing future studies. The information presented in this paper regarding NMES frequency and its cumulative effects will inform a subsequent clinical study that aims to assess the use of NMES in hypofunctional voice disorders.

CONCLUSION

This multi experiment study assessed changes in glottal configuration and voice parameters for different frequencies of NMES and during 12 minutes of NMES stimulation at 35 pps. Muscle activation varied across NMES frequencies with regards to level of activation and method. Lower frequencies produced stronger activation via muscle twitches, whilst higher frequencies produced weaker and smoother activation of muscles. Image analysis confirmed that significant changes in glottal configuration were more pronounced for lower frequencies. Progressive changes in glottal adduction and stiffness towards hyperfunction were produced within less than three minutes of stimulation. NMES also produced the activation of the CT muscles and the supraglottic structures and increased SPL. Therefore, shorter sessions of low frequency NMES with modified electrodes may be a safe and valuable resource for the treatment of hypofunctional voice disorders. Although this study highlights significant effects of NMES on laryngeal configuration, its use, combined with conventional voice therapy has the prevailing support in the literature and should be adopted in clinical practice. Considering the above results and in light of data from previous studies, the development of a clear guideline for NMES aimed at the treatment of hypofunctional voice disorders is required.

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