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Title

Postural strategy and back muscle oxygenation during inspiratory muscle loading

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Running title

Postural strategy during inspiratory muscle loading

Conflict of Interest and Source of Funding

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ABSTRACT

Purpose: Most healthy individuals show a multi-segmental control strategy during standing balance, whereas others show an ankle-steered strategy which is assumed as suboptimal. Respiratory demanding tasks exert a perturbing effect on balance, although the underlying mechanisms remain poorly understood. The purpose of this study was to investigate whether inspiratory resistive loading (IRL) affects postural strategy, back muscle oxygenation and blood volume during postural control.

Methods: We assessed the acute effects of increased respiratory effort by measuring center of pressure displacement in 12 healthy individuals during upright standing on an unstable support surface, whilst breathing against an IRL. Simultaneous ankle and back muscle vibration was used to evaluate the proprioceptive strategy (multi-segmental versus ankle-steered) during postural control. Back muscles oxygenation and blood volume were assessed using near-infrared spectroscopy (NIRS) (tissue oxygenation index (TOI) and combined haemoglobin (cHb)).

Results: An increased proprioceptive gain at the ankles and decreased gain at the back was observed after ~7 minutes of IRL. Retrospectively, the group was subdivided based on participants’ dominant proprioceptive use during baseline postural control. During IRL the ankle-steered group showed an increased reliance on ankle proprioception, compared to a multi-segmental group (-5.9±3.1cm and 1.0±1.9cm, respectively, p< 0.05). Both TOI and cHb declined progressively in the ankle-steered group during the IRL (-5.2±3.1% and -2.36±1.6µM, respectively, p< 0.05), whereas no decline was found in the multi-segmental group (-0.9±4.2% and 0.34±0.4µM, respectively, p> 0.05).
Conclusion: Individuals who adopted an ankle-steered strategy during IRL showed a progressive decline in back muscle oxygenation and blood volume. In contrast, IRL did not affect back muscle oxygenation and blood volume in individuals who showed a multi-segmental strategy in upright standing.

KEY WORDS Respiration, near-infrared spectroscopy, proprioception, sensory weighting, metaboreflex
INTRODUCTION

Paragraph number 1 Postural control is an essential requirement in physical and daily activities. Proprioceptive input is of primary importance in the control of postural balance (28). Optimal upright standing requires multi-segmental control at the ankles, knees, hips and spine (1). In posturally challenging situations, like standing on an unstable support surface, ankle proprioception becomes less reliable and consequently, the central nervous system is forced to use more proximal proprioceptive signals to provide optimal postural control, a process known as proprioceptive reweighting (21). However, in the presence of pain, fatigue or injury, specific proprioceptive signals may lose reliability. For example, when back proprioceptive signals lose reliability due to low back pain, individuals adopt an ankle-steered strategy, and the normal proprioceptive reweighting becomes compromised (7). Consequently, the ability of individuals with low back pain to switch to another postural strategy is impaired (10). Therefore, the use of an ankle-steered strategy, rather than multi-segmental control, is assumed as suboptimal during postural balance, since it reduces the versatility in postural strategies. The physiological mechanisms of this reduced postural versatility remain unclear.

Paragraph number 2 Respiration imposes a perturbing influence upon postural control (9, 27). Generally, healthy individuals are capable of compensating actively for this perturbation (20). However, postural compensation to respiratory perturbation becomes challenged when respiratory demand increases, for example during deep breathing (16, 20), voluntary hyperventilation (13, 35), and inspiratory resistive loading (IRL) (22). Moreover, IRL induces the use of a suboptimal ankle-steered strategy during postural control (22). The underlying
mechanisms for the apparent association between respiratory demands tasks and postural control remain to be examined systematically.

**Paragraph number 3** There is now ample evidence that high levels of inspiratory muscle work induce a generalized increase in sympathetic discharge that has been linked to the exacerbation of muscle fatigue (33). The underlying mechanism for this response is the activation of an inspiratory muscle metaboreflex by accumulating metabolites within the inspiratory muscles. This metaboreflex restricts locomotor muscle blood flow, due to preferential perfusion of the respiratory muscles (29, 34). Near-infrared spectroscopy (NIRS) is a noninvasive method to study regional muscle blood flow and oxygen utilization in real time (17), thereby assessing vasodilatation and vasoconstriction in skeletal muscles (15). Previous studies demonstrated changes in limb muscle oxygenation and blood flow during loading (26, 29), and unloading the inspiratory muscles (5). Since there is evidence that postural control is suboptimal when the inspiratory muscles are at risk of fatigue (13, 22), we may speculate that the metaboreflex may also impair the muscles involved in spinal control (6), for example the back muscles (23, 36). However, to the author’s knowledge, no study investigated the effect of increased inspiratory muscle work on back muscle oxygenation and blood flow. It remains unknown whether IRL affects back muscle oxygenation and blood flow which may induce a suboptimal postural strategy.

**Paragraph number 4** Therefore, the main objective of this study was to investigate whether IRL affects proprioceptive (re)weighting, back muscle oxygenation and blood volume during postural control. We hypothesize that IRL will necessitate the adoption of a suboptimal ankle-steered postural strategy, and that the concomitant activation of the inspiratory muscle metaboreflex will
lead to reduced back muscle oxygenation and blood volume. Postural control will be determined by standing on an unstable support surface (21), while breathing against an IRL. At the same time, simultaneous ankle and back muscle vibration will be used to evaluate the role of proprioceptive signals since it is a powerful stimulus of muscle spindle Ia afferents (7, 11, 32), and NIRS will be used to assess dynamic changes in back muscle oxygenation and blood flow (17, 24). To examine the contribution of habitual postural strategy, individuals who usually adopt an ankle-steered strategy will be compared with those who employ a multi-segmental strategy during postural control, presuming habitual performance may be predictive for future pathology.
METHODS

Subjects

**Paragraph number 5** Twelve healthy individuals (age: 21 ± 2 years, body mass index: 21 ± 2 kg/m²), participated voluntarily in this study. Individuals with a history of specific balance problems (e.g. vestibular or neurological disorder), respiratory problems, smoking, previous spinal surgery, lower limb problems, low back pain or the use of pain relieving medication or physical treatment were excluded. None of the subjects showed evidence of respiratory obstruction upon examination of forced expiratory volume in one second (FEV₁: 3.90 ± 0.72 l) and forced vital capacity (FVC: 4.72 ± 1.19 l) (2). All individuals showed normal respiratory muscle force, as assessed by maximal inspiratory pressure (PImax: 105 ± 16 cmH₂O) and maximal expiratory pressure (PEmax: 158 ± 39 cmH₂O) using an electronic pressure transducer (MicroRPM, Micromedical Ltd., Kent, UK). The Plmax was measured at residual volume and the PEmax at total lung capacity according to the method of Black and Hyatt (3). All participants gave their written informed consent conform to the principles of the Declaration of Helsinki (1964). The study was approved by the local Ethics Committee of Biomedical Sciences, KU Leuven, Belgium, and registered at www.clinicaltrials.gov with identification number NCT01541020.

**Proprioceptive weighting during postural control**

**Paragraph number 6** Postural sway characteristics were assessed by center of pressure (CoP) displacement using a 6-channel force plate (Bertec, OH) which recorded the moment of force
around the frontal axis (Mx) and the vertical ground reaction force (Fz). Force plate signals were sampled at 500 Hz using a Micro1401 data acquisition system using Spike2 software (Cambridge Electronic Design, UK) and were filtered using a low pass filter with a cut-off frequency of 5 Hz.

**Paragraph number 7** Local muscle vibration was used to investigate the role of proprioception in postural control. Muscle vibration is a powerful stimulus of muscle spindle Ia afferents (11, 32). It evokes an illusion of muscle lengthening in standing. If the central nervous system uses proprioceptive signals of the vibrated muscles for postural control, it will cause a directional corrective CoP displacement. When the triceps surae muscles (TS) are vibrated, a postural sway in a backward direction is expected, whereas during lumbar paraspinal muscle (LP) vibration, a forward postural body sway is expected, which has been shown by previous studies (7, 10, 22, 23). The amount of CoP displacement during local vibration may represent the extent to which an individual makes use of the proprioceptive signals of the vibrated muscles to maintain the upright posture. Simultaneous vibration on TS and LP muscles may identify the individual’s ability to gate conflicting proprioceptive signals (ankle versus back) during postural control (8). During simultaneous TS-LP muscle vibration, a dominant backward body sway suggests an ankle-steered postural control whereas a forward body sway indicates a more multi-segmental strategy. Muscle vibrators (Maxon motors, Switzerland) were applied bilaterally over the TS and LP muscles and vibration was offered at a high frequency and low amplitude (60Hz, 0.5mm) (32). Prior to the actual measurements subjects were presented with a few seconds of vibration in order to avoid startle effects.
Inspiratory resistive loading

Paragraph number 8 An IRL protocol was conducted using an electronic loading device (MicroRMA, MicroMedical Ltd, Kent, UK). The device imposes a constant resistance to inspiratory airflow by manipulating the surface area of the inspiratory airway. A week before the actual experiment, all participants performed a standard breathing trial with the workload increased by 2 kPa/l/sec every 7 breaths (incremental loading protocol). Participants were instructed to inhale against the IRL at a frequency of 15 breaths/min, duty cycle of 0.5 and a target flow of 0.6 l/sec until the flow could no longer be maintained. In addition, the participants were asked to perform a breathing trial with a constant preset inspiratory resistance of 70% of the maximum workload reached during the incremental loading protocol. This constant loading protocol was used as the definitive IRL protocol to examine the effect on proprioceptive postural control and back muscle parameters. The breathing trials were performed with the subject wearing a nose clip and breathing through the IRL device for maximum 900 seconds. An adapted Borg scale (0-10) was chosen to evaluate the respiratory effort during IRL (4). Both the incremental loading protocol as the definitive constant loading protocol were repeated twice and on different days in order to familiarize with the protocol before the actual test was performed.

Back muscle oxygenation and blood volume

Paragraph number 9 The back muscles are primarily involved in postural control (23, 37). Local muscle oxygenation profiles of the left LP muscles were evaluated using NIRS (NIRO 200NX, Hamamatsu Photonics, Japan) at two specific wavelengths (760 and 860 nm). The interoptode spacing (between emitter and detector) was 4 cm. The optical probes were firmly attached to the
skin at the LP muscles approximately 2 cm lateral to the spinous processes at the level of L3 (24). The NIRO 200NX provides a tissue oxygenation index (TOI) (expressed in %) and relative changes in oxyhaemoglobin (O2Hb) and deoxyhaemoglobin (HHb) (expressed in ΔµM.cm) (17). The sum of O2Hb and HHb indicates the change in total blood volume (combined haemoglobin: cHb), while the TOI value (O2Hb/cHb) is a measure of oxygen consumption. Respectively, a modified Beer-Lambert Law and Spatially Resolved Spectroscopy were used in the calculation of these parameters. To improve inter-subject comparability, values in TOI and cHb were expressed as the change from baseline.

**Experimental protocol**

*Paragraph number 10* During the entire experiment, the participants were instructed to stand barefoot on a foam pad, placed on the force plate, with their arms relaxed along the body. On unstable support surface (Airex balance pad; 49.5 cm length x 40.5 cm width x 6.5 cm thickness), ankle proprioceptive signals become less reliable which enforces reliance upon proximal proprioceptive signals, thereby highlighting proprioceptive deficits (21). A standardized foot position was used, with the heels placed 10 centimeter apart, and a free forefoot position. The vision of the subjects was occluded by means of non-transparent goggles. They were instructed to maintain their balance at all times during the experiment and an investigator was standing next to the subject to prevent actual falls.

*Paragraph number 11* Two experimental trials were implemented: 1) The first trial evaluated the weighting of proprioceptive input for postural control during quiet standing. Muscle vibration was applied bilaterally for 15 seconds to the TS muscles and LP muscles simultaneously. Before
the second trial started, the subjects were asked to move their lower limbs and pelvis briefly to reset muscle spindles. 2) During the second trial, the subjects were asked to perform the IRL protocol during simultaneous vibration on TS and LP muscles. Back muscle NIRS was evaluated during the whole trial. The end of the trial was reached by failure of the subject to reach a flow of 0.6 l/sec despite encouragement of the investigator. For pragmatic reasons, the maximum length of the second trial was set at 900 seconds. Figure 1 displays the experimental set-up of Trial 2.

***Please insert Figure 1 near here***

Data reduction and statistical analysis

Paragraph number 12 Force plate data and NIRS parameters were calculated using Spike2 software and Microsoft Excel. To evaluate the directional effect of muscle vibration, mean values of CoP displacement (anterior-posterior) were calculated by using the equation: CoP= Mx/Fz. Back muscles oxygenation (TOI) and blood volume (cHb) were calculated by using the equation: TOI= O2Hb/cHb and cHb= O2Hb+HHb, respectively. To investigate the capacity to compensate for IRL, mean CoP displacement and mean changes in TOI and cHb were calculated every 30 seconds. Mean values indicate the change compared to baseline (i.e., upright standing on unstable support surface without vision) before the start of muscle vibration (Trial 1) and IRL + muscle vibration (Trial 2). Positive values indicate an anterior body sway (CoP), increased back muscles oxygenation (TOI) and blood volume (cHb) whereas negative values indicate a posterior body sway (CoP), decreased back muscles oxygenation (TOI) and blood volume (cHb) compared to baseline. A dominant anterior body sway (CoP) suggests a multi-segmental strategy whereas a backward body sway indicates a more ankle-steered strategy during postural control (7, 10, 22, 23).
A repeated measures analysis of variance (ANOVA) was used to examine differences between subjects and within-subjects over time (each 30 seconds of IRL). A post hoc test (Fisher) was performed to further analyze these results in detail. A median split analysis was used to subgroup the individuals based on Trial 1, placing the participants with the most anterior body sway into the “multi-segmental group” and the participants with the most posterior body sway in the “ankle-steered group”. Chi-square statistics were used for nominal data. The statistical analysis was performed with Statistica 9.0 (Statsoft, OK, USA). The level of significance was set at p< 0.05.


RESULTS

Proprioceptive postural control during inspiratory resistive loading

Paragraph number 14 The group of healthy individuals showed a slightly posterior body sway during IRL combined with simultaneous TS-LP vibration (Figure 2). Moreover, this posterior sway increased progressively, and significantly, at ~450 seconds during the subsequent minutes of IRL. To explore this increased posterior sway, the group was retrospectively subdivided based on participants’ dominant use of proprioceptive signals (ankle versus back) during postural control. Two subgroups were defined based on the participant’s habitual postural control without IRL (Trial 1) placing the participants with the most anterior body sway into the “multi-segmental group” (-0.5 ± 1.1 cm) and the participants with the most posterior body sway in the “ankle-steered group” (-6.5 ± 4.4 cm) (F(1, 10)= 10.26, p= 0.009). Characteristics of two subgroups are presented in Table 1.

***Please insert Table 1 near here***

Paragraph number 15 During IRL combined with simultaneous TS-LP vibration, the ankle-steered group showed a significantly larger posterior sway compared to the multi-segmental group (F(1, 10)= 2.30, p= 0.000). This significant difference was present at all time points starting from the first 30 seconds, with the exception at 780 and 810 seconds. Figure 3 displays the mean CoP displacements each 30 seconds throughout the IRL protocol in the two subgroups. The duration of the IRL protocol did not significantly differ between the ankle-steered group (630 ± 245 s) and the multi-segmental group (750 ± 300 s) (F(1, 10)= 0.58, p= 0.465). However, only one participant from the ankle-steered group attained the maximum time of 900 seconds, compared with four from the multi-segmental group (χ= 3.09, p= 0.079). The workload and
perceived effort did not differ significantly between the ankle-steered group (13.4 ± 5.7 kPa/l/s and 8.8 ± 0.8, respectively) and the multi-segmental group (17 ± 5.1 kPa/l/s and 6.8 ± 2.7, respectively) (F(1, 10)= 1.31, p= 0.279 and F(1, 10)= 3.03, p= 0.112, respectively).

***Please insert Figure 2 near here***

***Please insert Figure 3 near here***

**Back muscle oxygenation and blood volume during inspiratory resistive loading and postural control**

**Paragraph number 16** Back muscles oxygenation (TOI) decreased significantly in the ankle-steered group compared to the multi-segmental group throughout the IRL protocol (F(1, 30)= 1.82, p= 0.007). The back muscles TOI showed no decline in the multi-segmental group during the full IRL (p= 0.37) whereas the ankle-steered group showed a significantly progressive decline during the IRL protocol (p= 0.000). After 5 minutes of IRL a significant difference between the groups was observed until task failure (p< 0.05).

**Paragraph number 17** Simultaneously, blood volume in the back muscles (cHb) decreased significantly in the ankle-steered group compared to the multi-segmental group (F(1, 30)= 7.95, p= 0.000). Whilst no decline was observed in the multi-segmental group (p= 0.167), back muscle blood volume decreased in the ankle-steered group during IRL (p= 0.000). A significant difference between the groups was observed after 2.5 minutes of IRL (p< 0.05). Figure 4 and 5 display the back muscles TOI and cHb, respectively, in the two subgroups during the IRL protocol.

***Please insert Figure 4 near here***

***Please insert Figure 5 near here***
DISCUSSION

**Paragraph number 18** Inspiratory resistive loading forces healthy individuals to switch to a suboptimal postural control strategy with an increased proprioceptive gain at the ankles and a decreased gain at the back muscles after ~7 minutes of IRL. Individuals who showed an increased reliance on ankle proprioceptive signals during postural control in the baseline condition, maintained this suboptimal postural strategy during IRL. Additionally, during IRL this ankle-steered group showed a progressive decline in back muscle oxygenation and blood volume. In contrast, individuals who showed a more multi-segmental control in baseline upright standing maintained this optimal postural strategy during IRL while back muscle oxygenation and blood volume were not affected. Although the effect of inspiratory muscle work has been widely examined on limb muscle oxygenation and blood flow affecting cycling and running performance (5-6, 29, 33-34), this was the first study examining the effect of inspiratory muscle work on back muscle oxygenation and blood flow during a postural control performance.

**Paragraph number 19** Muscle blood volume and oxygenation are determined by the summation of a number of inputs to the muscle vasculature, some of which exert a vasoconstrictor influence, whilst others induce vasodilatation. These inputs include efferent sympathetic vasoconstrictor activity and the vasodilator influence of local muscle metabolites (14). Inspiratory resistive loading is known to elicit a generalized increase in sympathetic outflow, which induces vasoconstriction in locomotor muscles (i.e., metaboreflex) (29, 33-34). However, in the presence of muscle activity, this neutrally-mediated vasoconstrictor influence may be successfully opposed by local vasodilator influences (14). Our results show a decreased back muscle oxygenation and blood volume in the ankle-steered group, but not in the multi-segmental group. This observation is consistent with a mechanism whereby back muscle blood volume was maintained in the multi-
segmental group by the vasodilator influence of local back muscle metabolites. This vasodilator influence was absent in the ankle-steered group, presuming decreased back muscle use to maintain postural control (23, 36). Since muscles spindles show a dense capillary system, the inability to counteract the vasoconstrictor influence of the metaboreflex, will inevitably affect muscle spindle function (25). The vasoconstriction within the back muscles of the ankle-steered group may induce a decrease in back muscle spindle sensitivity. This may necessitate the central nervous system to downweight back proprioceptive signals for postural control and consequently adopting an even more ankle-steered postural control strategy during IRL (7, 10, 22). Accordingly, our findings may imply that loading of the inspiratory muscles negatively affects proprioceptive postural control, possibly via the inability to counteract back muscle vasoconstriction imposed by the inspiratory muscle metaboreflex.

**Paragraph number 20** The occurrence of a metaboreflex may explain the abrupt increase in posterior sway in the total group around 450 seconds of IRL (Figure 2). However, IRL seemed to force individuals to reweight their proprioceptive signals and change their postural control strategy at the commencement of IRL (Figure 3). This strategy might be explained by the Central Governor Model, an anticipatory regulatory model that allows feedback from the periphery to influence the feedforward central drive that determines the extent of specific muscle recruitment (30). According to this model, independent systems in the periphery provide sensory feedback that influences central motor drive from the brain to the exercising muscles to ensure that a certain task performance (e.g., postural control) changes before a biological ‘failure’ appears. This model is consistent with our observation that the ankle-steered group adopted a posterior body sway at the start of IRL although the effects of the metaboreflex would only occur progressively during the exercise. The dual engagement of the inspiratory muscles, both in IRL
and postural control (18), might overload the multi-segmental control performance and consequently forces an alternative suboptimal ankle-steered strategy to be used.

**Paragraph number 21** Using proprioceptive reweighting, the central nervous system increases the proprioceptive gain at the ankle muscles when the back muscle proprioceptive signals lose reliability, e.g. in individuals with low back pain (7, 10), or in back muscle fatigue (23, 37). Previous studies demonstrated increased ankle muscle activity (12, 13), and increased lower limb nerve excitability (35) in postural control during hyperventilation, but did not examine more proximal proprioceptive sources like the back trunk muscles, although assumed essential for optimal postural control (1, 36). Recent evidence showed that a flexible multi-segmental control, and not a stiff strategy, is preferred to compensate for breathing (27). Furthermore, a recent study revealed an increased use of ankle proprioceptive signals in postural control immediately after IRL (22). However, to our knowledge, the present study is the first to examine proprioceptive weighting changes in postural control during IRL. In this respect, simultaneous vibration of TS and LP muscles provides a useful tool to examine dynamic changes in proprioceptive weighting during physiological perturbations such as IRL.

**Paragraph number 22** Our findings have potential clinical relevance for respiratory demanding sports involving high amounts of walking and running, since these sports are likely to reduce postural control (31). The use of an ankle-steered strategy following inspiratory muscle loading may increase the risk of sports injuries, either by a higher fall risk or development of low back pain associated with decreased trunk control (19). Accordingly, interventions such as inspiratory muscle training may have a positive effect on postural balance, and warrant further exploration.
Paragraph number 23 To provide definitive evidence of the inspiratory muscle metaboreflex, we suggest future studies recording blood pressure and heart rate during IRL. In addition, transdiaphragmatic pressures and evoked potential responses to bilateral phrenic nerve magnetic stimulation would reveal the presence, or otherwise, of contractile fatigue of the diaphragm (2).

Paragraph number 24 In conclusion, loading of the inspiratory muscles forced healthy individuals to shift to a suboptimal postural strategy during upright standing, with an increased gain at the ankles and a decreased use of back muscle proprioceptive signals. This downweighting of back proprioceptive signals was associated with a decreased back muscle oxygenation and blood flow. This might suggest a decreased ability to counteract the vasoconstrictor influence of the inspiratory muscle metaboreflex upon back muscle oxygenation and blood volume. The latter may impair the reliability of proprioceptive signals from the back muscles, thereby necessitating the central nervous system to adopt a suboptimal, ankle-steered postural control strategy. Our findings provide a possible explanation for the reduced postural control and spinal injuries observed in high intensity sports. Further studies must reveal whether unloading of the inspiratory muscles might have a positive effect on proprioceptive postural control.
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CONFLICT OF INTEREST

Alison McConnell acknowledges a beneficial interest in an inspiratory muscle training product in the form of a share of license income to the University of Birmingham and Brunel University. She also acts as a consultant to POWERbreathe International Ltd.
REFERENCES (max 40)


FIGURE LEGENDS (max 6 Figures & Tables in total)

**Figure 1.** Experimental set-up (Trial 2): inspiratory resistive loading during standing on an unstable support surface with combined vibration of triceps surae and lumbar paraspinal muscles.

**Figure 2.** Center of pressure displacement (mean ± SD) in the total group during inspiratory resistive loading (IRL) and combined vibration of triceps surae and lumbar paraspinal muscles (TS-LP). Positive values indicate an anterior body sway, negative values indicate a posterior body sway compared to baseline. Significant differences are found between 450 seconds and 510, 540, 570, 600, 630, 660, 840 and 870 seconds, respectively, which indicate an increased posterior sway starting from 450 seconds of IRL. (* = p< 0.05)

**Figure 3.** Center of pressure displacement (mean ± SD) in the multi-segmental group (black) and ankle-steered group (white) during inspiratory resistive loading (IRL) and combined vibration of triceps surae and lumbar paraspinal muscles (TS-LP). Positive values indicate an anterior body sway, negative values indicate a posterior body sway compared to baseline. (* = p< 0.05)

**Figure 4.** Back muscles Tissue Oxygenation Index values (TOI) (mean ± SD) in the multi-segmental group (black) and ankle-steered group (white) during inspiratory resistive loading (IRL) and combined vibration of triceps surae and lumbar paraspinal muscles (TS-LP). (* = p< 0.05)
**Figure 5.** Back muscle combined Haemoglobin values (cHb) (mean ± SD) in the multi-segmental group (black) and ankle-steered group (white) during inspiratory resistive loading (IRL) and combined vibration of triceps surae and lumbar paraspinal muscles (TS-LP). (* = p< 0.05)