Physiological responses and welfare implications of rapid hypothermia and immobilization with high levels of CO_2 at two temperatures in Arctic char (*Salvelinus alpinus*).

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Running title: Stress responses to hypothermia and carbon dioxide in char

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Summary

Carbon dioxide (CO_2) is used for immobilization of Arctic char (*Salvelinus alpinus*) prior to slaughter at Swedish aquaculture facilities, and fish are routinely exposed to hypothermia in ice water during transport. Yet, information on stress physiological responses to CO_2, temperature extremes and their potential interacting effects is scarce for this cold-water species. Here, blood pressure, heart and ventilation rates and plasma variables including ions, haematocrit, glucose and cortisol were measured in cannulated char during exposure to hypothermia (i.e. a rapid temperature drop from 10°C to 0.25°C); as well as to water nearly saturated with CO_2 at 10°C and 0.25°C to test the hypothesis that hypothermia alleviates stress responses during CO_2 exposure. While all fish maintained equilibrium during the 30 min hypothermic challenge, blood pressure, heart and ventilation rates decreased and plasma cortisol increased moderately. CO_2 exposure at 10 and 0.25°C resulted in aversive behavioural reactions before equilibrium was irrecoverably lost after 184±14 and 191±9 seconds, respectively. The physiological responses to CO_2 exposure were largely similar at both temperatures with elevated cortisol levels, reduced heart and ventilation rates and hypotension; although reductions in ventilation amplitude and arterial pulse pressure were significantly more pronounced at 0.25°C. It is concluded that hypothermia alone is a relatively mild stressor in this species, while CO2 exposure elicits pronounced physiological and behavioural stress responses that are not alleviated by hypothermia.

Keywords: blood pressure, carbon dioxide, cortisol, heart rate, haematology, ventilation.

Introduction

High levels of carbon dioxide (CO_2) are still widely used to immobilize fish prior to slaughter at many aquaculture facilities (EFSA, 2009; Robb, et al., 2000; van de Vis, et al., 2003). The use of this method mainly relates to the low costs and ease of use, in combination with the advantage that CO_2 leaves no toxic residues in fish produced for human consumption. Nevertheless, serious concerns have been expressed regarding the welfare implications of CO_2 anaesthesia as the method typically results in a pronounced primary stress response including release of cortisol and catecholamines (Bernier and Randall, 1998; Iwama, et al., 1989; Wagner, et al., 2002). Struggling behaviours and other aversive reactions indicative of stress and compromised welfare have also been frequently reported (EFSA, 2009; Erikson, 2011; Marx, et al., 1997; Robb, et al., 2000; Roth, et al., 2002; van de Vis, et al., 2003). Consequently, alternative methods or means of refining existing methods to immobilize fish are widely sought after by the industry.

Combinations of hypothermia (also referred to as live-chilling) and CO_2 have been used to sedate Atlantic salmon (*Salmo salar*) prior to slaughter in large scale Norwegian salmon processing facilities (Erikson, 2008; Erikson, et al., 2006). In addition, ice water is sometimes used during transport prior to slaughter to reduce the metabolism and activity of the fish to improve meat quality by extending the time to onset of muscle rigor (Erikson, 2011; Erikson, et al., 2006; Jittinandana, et al., 2005; Roth, et al., 2002; Skjervold, et al., 1999; Skjervold, et al., 2001). Hypothermia has been suggested to be beneficial from an animal welfare perspective as it reduces crowding stress and may reduce physiological stress responses (Erikson, et al., 2006; Skjervold, et al., 2001). For example, hypothermia could alleviate the stress effects during immobilization with high levels of CO_2 by reducing metabolic rate; thereby reduce the hypoxemia caused by haemoglobin Root and Bohr shifts as the blood pH drops (Dejours, 1975). In fact, Yokoyama et al. (1989) suggested that the

efficacy of long-term CO_2 anaesthesia increased when the temperature was reduced in carp (*Cyprinus carpio*). On the other hand, rapid temperature reductions *per se* (i.e. cold shock) may result in primary and secondary stress responses in fish, including elevated plasma levels of cortisol and catecholamines, suggesting that the physiological responses to hypothermia are highly context- and species-specific (Barton, et al., 1985; Chen, et al., 2002; Donaldson, et al., 2008; Foss, et al., 2012; Hyvärinen, et al., 2004; Tanck, et al., 2000).

The Arctic char is an increasingly important aquaculture species in northern Sweden, partly due to a successful breeding program of the fast growing strain 'Arctic superior' (Eriksson, et al., 2010). CO_2 is still widely used to immobilize char prior to slaughter at small and medium-sized facilities, and slaughter may take place throughout the year at varying water temperatures. Furthermore, live transport of char is routinely performed in tanks with ice water exposing fish to hypothermic challenges (personal observations). However, despite its growing commercial importance, knowledge about the stress physiology of the char is very limited compared with other salmonids. While the Arctic char is generally considered to be a 'cold-water' species with the most northerly geographical distribution of all salmonids (Fletcher, et al., 1988; Hovda and Linley, 2000), the physiological responses to acute hypothermia as well as the interacting effects of CO_2 exposure and temperature has to our knowledge not been examined.

In this study, physiological responses indicative of stress were evaluated in Arctic char (*Salvelinus alpinus*) in response to hypothermia alone (i.e. a rapid temperature drop from 10°C to 0.25°C) and during exposure to maximum levels of CO_2 at 10 and 0.25°C. First, cardioventilatory variables and physiological blood variables were monitored in chronically cannulated fish during rapid hypothermia in normoxic water to allow a comparison of cardioventilatory and blood physiological responses with other fish species (see Donaldson, et al., 2008). Second, the possibility of using hypothermia as a simple and economic means to

alleviate physiological stress responses and reduce induction times when immobilizing char with CO_2 was evaluated. The latter was specifically designed to mimic conditions at small scale aquaculture facilities where fish are typically rapidly exposed to CO_2 saturated water (EFSA, 2009; Robb, et al., 2000; van de Vis, et al., 2003).

Material and methods

Animals

All experiments were performed according to national legislation and ethical permit #149-2010 approved by the animal ethics committee of Gothenburg. Arctic char (*Salvelinus alpinus*) in the size range 390-620 g (mean ± SE: 491±19 g) were obtained from Aquaculture Centre North, Kälarne, Sweden and transported by truck to the laboratory by a professional fish farmer. They were kept in tanks supplied with partly recirculating UV-treated and bio-filtered fresh water at 10°C for at least 4 weeks prior to experimentation. Fish were fed commercial pelleted feed and were held on a photoperiod of 12:12 h (light:dark).

Surgical instrumentation

Individual fish were anaesthetized in aerated water containing 100 mg l^{-1} of tricaine methane sulphonate (MS-222; Sigma-Aldrich, St Louis, USA) buffered with $NaHCO_3$ (200 mg l^{-1}) and placed on a surgery table covered with wet rubber foam. The gills were continuously irrigated via the mouth with aerated water (10°C) containing a lower dose of $NaHCO_3$ buffered MS-222 (50 mg l^{-1} and 100 mg l^{-1}, respectively). The dorsal aorta was cannulated through the roof of the buccal cavity with a PE-50 catheter using a sharpened steel wire guide (Axelsson and Fritsche, 1994), and filled with heparinised (~100 IU ml^{-1}) saline (0.9%). To measure ventilatory movements, a heat flared PE-90 catheter was attached to the operculum with the

flared lumen facing the opercular cavity (Holeton and Randall, 1967). The catheter was secured from the outside of the operculum with a ~5 mm sleeve of heat flared PE-190 catheter, and filled with water to record opercular cavity pressure changes. Catheters were collectively sutured to the skin at the back of the fish using silk sutures. All fish were given at least 24 h of post-surgical recovery at 10°C before experiments started. In some cases fish were placed immediately in the experimental tube, while in other cases it was first placed in a separate holding tube resembling the experimental tube after surgery, and then transferred to the experimental tube at least 12 hours before the experiments started using an opaque water-filled plastic bag.

Experimental setup

The experimental tubes were connected via three-way valves to the departmental water system and to a separate 250L barrel containing water prepared by gassing (gas flow: 6L min^{-1}) the water for at least 2 hours to near CO_2 saturation (pH 5.0-5.4) to mimic conditions typically employed in Swedish aquaculture facilities (personal observations). The water was maintained at either the holding temperature of 10°C, or chilled to 0.25°C (mean: 0.25±0.05°C) using a separate chilling unit. This setup allowed us to rapidly expose the fish (within ~30s) to nearly CO_2-saturated water at the two selected temperatures without confounding stress-effects imposed by handling of the fish (Foss, et al., 2012). During experiments, chilled and CO_2-treated water was not recycled but drained into the sewer system.

Experimental protocols

In order to examine the effects of rapid hypothermia and high CO_2 exposure, alone and in combination, individual fish were exposed to one of the following experimental protocols:

1. Fish (n=14) were exposed to a rapid (within 30s) decrease in temperature from 10°C to 0.25°C, which was maintained for 30 min in normoxic water to determine the effects of hypothermia alone. Blood samples were taken before exposure (0 min), and at the end of the 30 minute period of hypothermic exposure.

2. Fish (n=8) were exposed to CO_2-saturated water at 10°C. Blood samples were taken before exposure and after 10 min exposure.

3. Fish (n=8) were exposed to CO_2-saturated water at 0.25°C to study the effects of CO_2 anaesthesia in combination with rapid hypothermia. Blood samples were taken as in protocol 2.

Cardioventilatory variables were recorded continuously in all experimental protocols, but are reported as mean values at selected time points.

Acquisition of cardioventilatory variables and analytical procedures

Pressure catheters were connected to blood pressure transducers (model DPT-6100, pvb Medizintechnik, Kirchseeon, Germany) that were calibrated against a static water column and the water surface in the holding tube served as the zero pressure reference. A 4ChAmp pre-amplifier (Somedic AB, Hörby, Sweden) amplified the signals from the transducers. Data was sampled at 40 Hz using a Power Lab unit (ADInstruments Pty Ltd, Castle Hill, Australia) connected to a laptop computer running LabChart Pro software (version 7.2; ADInstruments Pty Ltd, Castle Hill, Australia). The pulsatile blood pressure signal was analyzed off-line and mean dorsal aortic blood pressure (P_{da}), diastolic pressure ($P_{da\ dia}$), systolic pressure ($P_{da\ sys}$),

pulse pressure ($P_{da\ pulse}$) and heart rate (f_H) were calculated using the blood pressure module in the LabChart Pro software. Similarly, ventilation rate (f_V) and ventilation amplitude (amp_V) were derived from the pulsatile traces from the branchial catheter (Holeton and Randall, 1967). Ventilation amplitude is expressed as a percentage of pre-treatment control values.

Blood samples (0.5ml sample^{-1}) were collected using heparinized syringes. A handheld i-STAT clinical blood analyzer was used to analyze: Na^+, K^+, free Ca^{2+} (iCa^{2+}) and glucose (Glu). Haematocrit (Hct) was determined using micro capillary tubes spun in a hematocrit centrifuge. Whole blood was spun and plasma was collected and stored at -80^{o}C. Subsequently, plasma cortisol was analyzed with the radioimmunoassay technique described by Young (1986) using a cortisol antibody (Code: S020; Lot:1014-180182) purchased from Guildhay Ltd. (Guildford, Surrey, U. K.) and validated by Sundh, et al. (2011). As tracer, hydrocortisone-[1,2,6,7-3H(N)] (NET 396, NEN Life Sciences Products, USA) was used and cortisol standards were prepared from hydrocortisone (Sigma, St. Louis,USA). The determination of the radioactivity, and thus cortisol levels, was performed with a Wallac 1409 liquid scintillation counter.

Statistical analysis

For the two CO_2 treatments, the time to loss of equilibrium (anaesthesia stage I) was calculated as the time from the beginning of the exposure period (Bernier and Randall, 1998). Changes in cardioventilatory and blood variables during hypothermic and CO_2 exposure were analyzed using repeated measures ANOVA followed by Dunnet's post-hoc test to identify significant changes from baseline. Treatment effects on cardioventilatory, blood physiological and behavioural variables were analysed using unpaired t-tests. Treatment effects were considered statistically significant at $p< 0.05$.

Results

Cardioventilatory responses and behaviour

Representative original cardiovascular and temperature traces for the hypothermic exposure are presented in Fig. 1. The temperature in the holding tubes dropped to the desired temperature (mean: 0.25±0.05˚C) within approximately 30 seconds. There were no obvious behavioural reactions to the rapid reduction in temperature as the fish typically remained motionless in the experimental tubes and all fish maintained equilibrium for the duration of the hypothermic exposure (see table 1). Routine f_H, P_{da} and f_V were 42.3±3.7 beats min^{-1}, 3.2±0.1 kPa and 62.6±3.2 beats min^{-1}, respectively in the group that was exposed to hypothermia (Fig 2). A brief bradycardia and hypertension were often observed during the first 10-20 seconds of the exposure, which were likely of neural reflex origin (Fig. 1). The beat-to-beat variability of the heart then typically stabilized and the mean heart rate, dorsal aortic blood pressure and ventilation rate decreased gradually and significantly as the hypothermic exposure progressed with f_H, P_{da} and f_V reaching final values of 22.4±2.1 beats min^{-1}, 2.3±0.1 kPa and 37.8±1.5 beats min^{-1}, respectively after 30 min exposure (Fig 2). The reductions in f_H and f_V represented Q_{10} values of 1.9 and 1.7, respectively. Associated with these immediate responses to the hypothermic exposure were significant increases in $P_{da\ pulse}$ and amp_V (Fig 2). Although the fish remained calm in the holding tubes when the temperature was restored to 10°C, a slight tachycardia, hypertension and increased ventilation frequency were observed 20 min after the hypothermic exposure.

Routine f_H, P_{da} and f_V were 42.2±5.7 beats min^{-1}, 3.1±0.1 kPa and 63.6±4.7 beats min^{-1}, respectively in the group that was exposed to high CO_2 alone; and 34.8±4.2 beats min^{-1}, 3.2±0.1 kPa and 62.0±4.0 beats min^{-1}, respectively in the group that was exposed to CO_2 combined with hypothermia (Fig 3). The exposures to high CO_2 elicited pronounced

struggling and escape behaviour, but there were no obvious differences in behaviour between exposure temperatures. The recorded time to loss of equilibrium during CO_2 exposure was roughly 3 min, and these values did not differ significantly between temperatures (see table 1). Pressure artefacts due to the struggling behaviour precluded any detailed analyses of the cardioventilatory responses during the initial phase of the CO_2 exposures. However, f_H, P_{da} and f_V were all significantly reduced after 5 and 10 min of exposure in the CO_2 exposed fish (Fig. 3). The most striking difference in response to CO_2 between temperatures was that the ventilation amplitude did not decrease significantly in the group maintained at 10°C, while it was significantly reduced in the hypothermic group. The arterial pulse pressure also decreased with exposure to CO_2; although it was not significantly reduced in the 10°C group after 5 min exposure and the reduction was more pronounced during hypothermia as revealed by a significantly lower $P_{da\ pulse}$ after 10 min exposure (Fig. 3).

Blood variables

Plasma cortisol values increased significantly from 27.5±3.5 before to 47.0±6.2 ng ml^{-1} at 30 min of hypothermic exposure. During CO_2 exposure at 10 and 0.25°C, plasma cortisol increased from 29.2±8.8 to 58.0±13.5 ng ml^{-1} and from 24.9±3.2 to 73.7±12.6 ng ml^{-1}, respectively (Fig. 4).

Blood physiological responses to hypothermia and CO_2 exposure are presented in table 2. The only significant haematological effect of hypothermia was a reduction in haematocrit after 30 min exposure. The exposure to CO_2 treated water caused substantial increases in haematocrit and plasma [K^+] at both temperatures. The latter was likely due to haemolysis as a darkening of the plasma was routinely observed. Plasma [Na^+] and glucose levels were only significantly reduced during CO_2 exposure at 0.25°C, but remained unchanged at 10°C (table 2).

Discussion

By instrumenting fish with dorsal aortic and branchial cannulae we were able to continuously record cardioventilatory variables and repetitively sample blood for determination of plasma cortisol levels and haematological variables. This approach allowed us to evaluate the physiological effects of the temperature and CO_2 treatments *per se* without the effects of handling stress, because previous studies in Atlantic salmon have indicated that stress from handling may override the effects of hypothermia (Foss, et al., 2012). While literature on cardiovascular data for char is scarce, routine heart and ventilation rates in the present study were somewhat lower than in a previous study using the same instrumentation on Arctic char, albeit from a different stock (Sandblom, et al., 2012). This difference may be explained by the slightly higher temperature in the previous study (13°C). Routine plasma cortisol levels in the present study (see Fig. 4) are also somewhat lower than those recorded in the previous study (~48 ng ml^{-1}) (Sandblom, et al., 2012).

Physiological responses to hypothermia

While several studies have examined the metabolic and cardioventilatory responses to rapid temperature increase in fish (see Farrell, et al., 2009), few have described the responses to rapidly induced hypothermia (see Donaldson, et al., 2008). The cardioventilatory responses largely followed the expected pattern with reductions in heart rate and ventilation rates with Q_{10} values of 1.9 and 1.7, respectively. The blood pressure also decreased, which was likely due to a reduction in cardiac output because blood viscosity and vascular resistance would be expected to increase with reduced temperature (Clark, et al., 2008; Fletcher and Haedrich, 1987; Graham and Fletcher, 1983, 1985). Furthermore, the gradual nature of the cardioventilatory responses suggest that they were the combined result of a direct depressant effect of the low temperature on cardiac and ventilatory pacemaker tissues and a reduced

metabolic rate as the body temperature gradually equilibrated with the surrounding water (Crawshaw, 1976; Stevens and Sutterlin, 1976). Interestingly, a slight hypertension and increased heart rate was observed when the temperature was returned to 10°C following the hypothermic exposure. While plasma catecholamines were not measured in the present study, this response may have been due to a remaining effect from elevated levels of plasma catecholamines, which are known to increase during acute hypothermia in fish (Chen, et al., 2002).

The preferred temperature of a lake population of Arctic char has been reported to vary from 8.7 to 11.8°C throughout the year (Mortensen, et al., 2007). This temperature range brackets the acclimation temperature of 10°C in the present study. Nevertheless, the steep reduction in temperature to 0.25°C examined here can probably be regarded as a relatively mild stressor for this species because no aversive reactions were observed upon exposure to hypothermia, and after 30 minutes of acute hypothermic exposure the plasma cortisol level had only increased from 27.5±3.5 to 47.0±6.2 ng ml^{-1}. This can be compared with other more severe stressors such as confinement stress and non-lethal electrical exposure where plasma cortisol levels can reach values of at least ~200 ng ml^{-1} (Pottinger, 2010; Sandblom, et al., 2012). However, future studies will have to reveal whether a longer hypothermic exposure, which might occur during live transport of char in ice water, results in more pronounced primary stress responses.

The acute hypothermic exposure did not cause any significant changes in any of the measured plasma ions (see table 2). This contrasts with several previous studies where decreases in plasma ions have been reported during rapid hypothermia, which is thought to be due to a greater thermal sensitivity of the metabolically demanding uptake than the passive efflux of ions in freshwater (Donaldson, et al., 2008; Gonzales and McDonald, 2000). However, a recent study on Atlantic salmon in saltwater indicated that handling stress

associated with transfer between temperatures is crucial for whether ionic imbalances occur during hypothermia because when salmon were exposed to a temperature drop from 16 to 4°C by physical transfer a significant increase in plasma [Na^+] occurred, while no such change was detected when the fish was subjected to the same temperature reduction without handling (Foss, et al., 2012). It is possible that the practice of exposing char to severe hypothermia without any associated handling stress and with minimal behavioural reactions in the present study might explain the lack of changes in plasma ion composition. Another possibility is that the cold water tolerant char is unusually tolerant to changes in temperature reductions with regard to ion homeostasis.

The only clear blood physiological response that was observed in response to hypothermia was a significant reduction in haematocrit. The haematocrit response to acute cold exposure appear to be quite variable in fish and is likely influenced by a complex interaction of e.g. exposure time, changes in plasma catecholamine levels, osmotic changes in the blood as well as splenic contributions (Donaldson, et al., 2008). The lack of changes in plasma ion concentration indicates that the reduced hematocrit was not due to plasma dilution during the hypothermic challenge, but the exact mechanism behind this response is presently unclear.

Stress responses during CO_2 exposure and welfare implications of hypothermia

The time to loss of equilibrium during CO_2 exposure was not significantly different between temperatures, suggesting that the time to loss of consciousness was not reduced by the hypothermic exposure. If anything, the primary stress response appeared somewhat greater in fish that were exposed to high levels of CO_2 at 0.25 than at 10°C because plasma cortisol levels tended to be higher in fish that were exposed to high levels of CO_2 at 0.25°C, although this trend did not reach the level of statistical significance due to great inter-individual

variability. Moreover, Na^+ decreased significantly during CO_2 exposure at 0.25°C and the haematocrit increase tended to be more pronounced at the low temperature (table 2), which collectively may indicate a greater catecholaminergic activation of red blood cell Na^+/H^+-exchangers to maintain intracellular pH homeostasis (Perry and Bernier, 1999).

There were dramatic reductions in heart and ventilation rates and a substantial drop in both the mean blood pressure and pulse pressure during exposure to the CO_2 treated water at both temperatures. These responses were probably signs of lethal cardioventilatory collapse caused by a severe acidosis. The severe nature of this treatment was further supported by the finding that none of the fish recovered when returned to normoxia after 10 minutes. However, an interesting observation in this regard was that ventilation amplitude decreased significantly during CO_2 exposure at 0.25, although CO_2 exposure at 10°C and hypothermia alone had no effect on ventilation amplitude. While the mechanism behind this difference is unclear, it highlights the unpredictable relationship between temperature and CO_2 exposure.

The strongly aversive behavioural reactions, as well as the primary and secondary physiological stress responses during CO_2 exposure in char in the present study is in line with previous studies on other species of fish (Bernier and Randall, 1998; Erikson, 2011; Iwama, et al., 1989; Marx, et al., 1997; Robb, et al., 2000; Roth, et al., 2002; Wagner, et al., 2002; van de Vis, et al., 2003). Thus, in addition to confirming that high levels of CO_2 represents a highly stressful means of immobilising and euthanizing fish, an important finding here is that an acute reduction in temperature does not have any positive effects on welfare in a cold water species like the Arctic char. Thus, while the practice of cooling fish in e.g. ice-slurries or cool water prior to CO_2 exposure may have beneficial implications on meat quality, the present study suggests that the benefits from an animal welfare perspective may in fact be the opposite.

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Figure legends

Fig. 1 Representative original traces of dorsal aortic blood pressure, temperature and heart rate in Arctic char (*Salvelinus alpinus*) during the initial phase of hypothermic exposure. Note the initial brief bradycardia and increase in blood pressure upon exposure to the hypothermic water (vertical arrows).

Fig. 2 Hemodynamic and ventilatory variables (n=13-14) in Arctic char (*Salvelinus alpinus*) before (0 min), during 5 and 30 min of hypothermic exposure (0.25°C) and at 20 min after hypothermic exposure when the temperature had been returned to 10°C. All values are means (+SEM). Asterisk denotes statistically significant difference ($p \leq 0.05$) from the 0 min value. All fish recovered from the hypothermic exposure.

Fig. 3 Hemodynamic and ventilatory variables (n=8) in Arctic char (*Salvelinus alpinus*) at 10°C before (0 min) and after 5 and 10 min of exposure to high CO_2 at 10°C (grey bars) or 0.25°C (black bars). All values are means (+SEM). Asterisk denotes statistically significant difference from the corresponding 0 min value and dagger denotes statistically significant difference between treatment temperatures at a given time point ($p \leq 0.05$). None of the fish recovered from the CO_2-exposures.

Fig. 4 Plasma cortisol levels (n=6-10) in Arctic char (*Salvelinus alpinus*) at 10°C before (control) and during exposure to hypothermia (0.25°C) and high CO_2 at 10°C or 0.25°C (exposure). All values are means (+SEM). Asterisk denotes statistically significant difference from the respective control value and dagger denotes statistically significant difference between treatments ($p \leq 0.05$).

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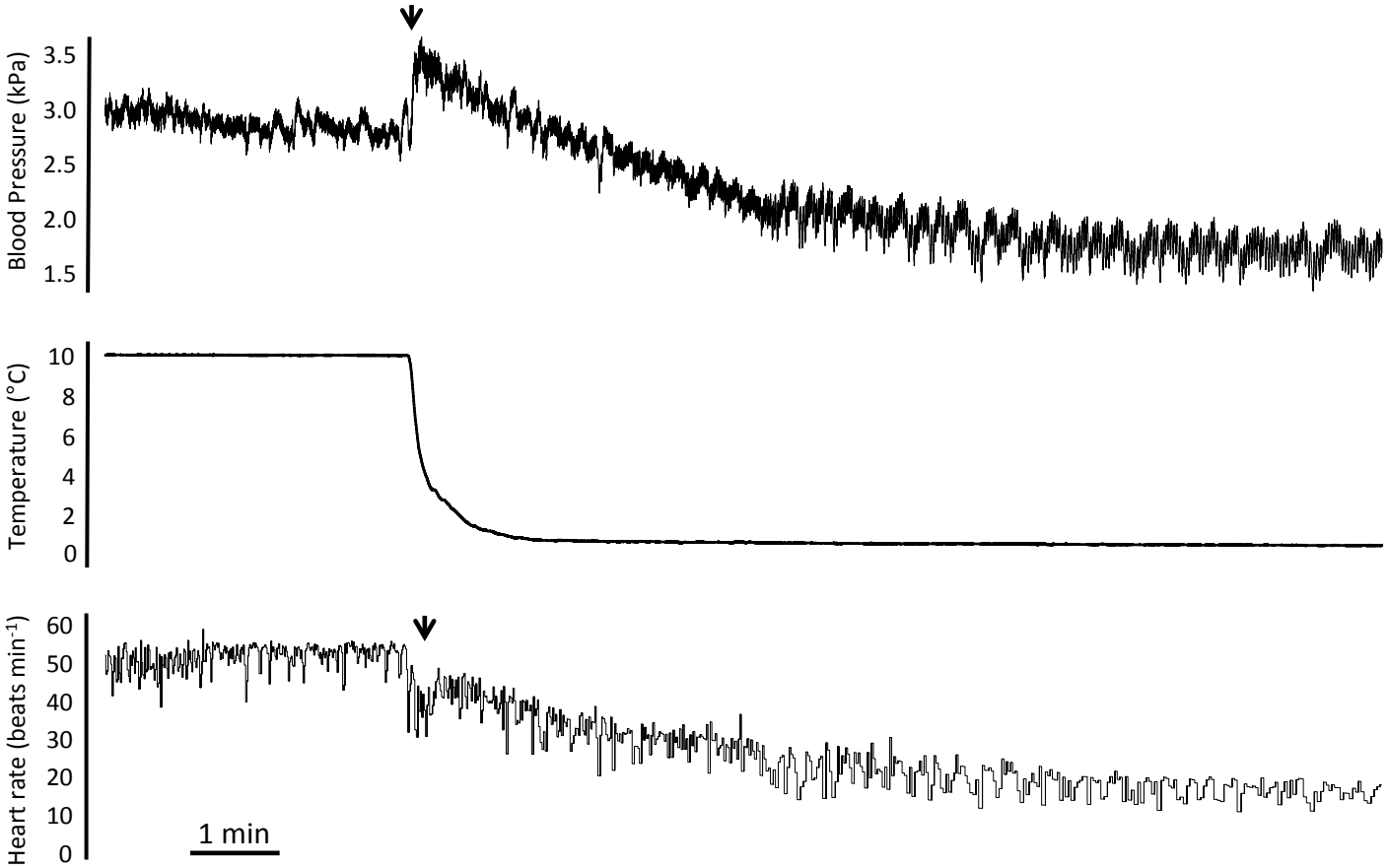
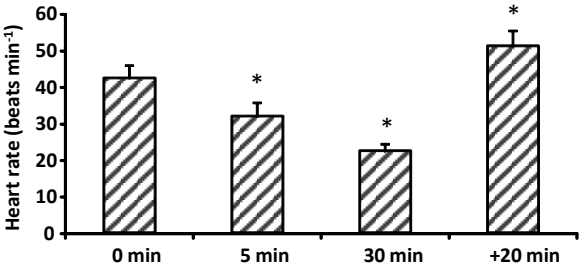
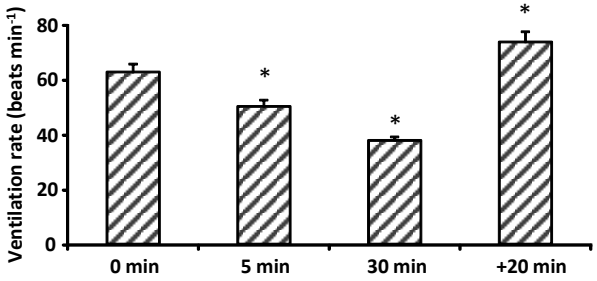
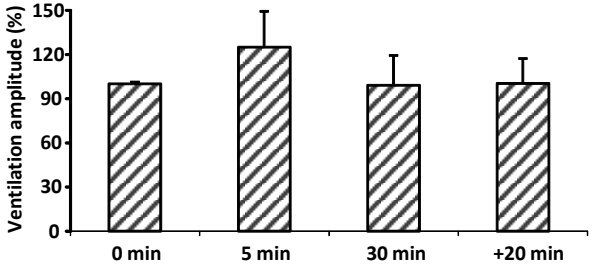
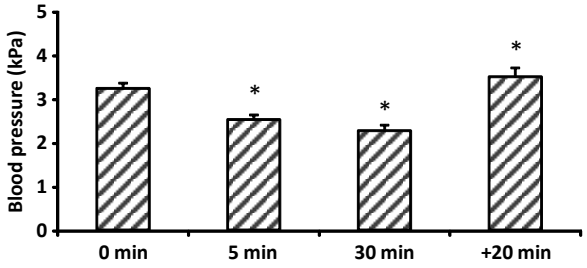
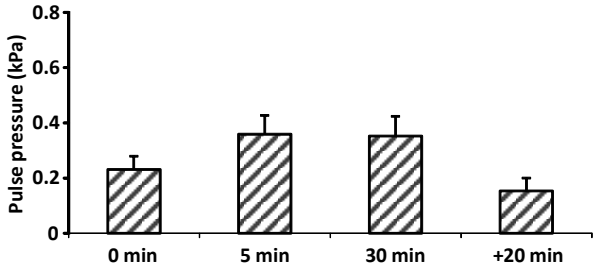

Blood Pressure (kPa)
3.5
3.0
2.5
2.0
1.5
Temperature (°C)
10
8
6
4
2
0
Heart rate (beats min-1)
60
50
40
30
20
10
0
1 min

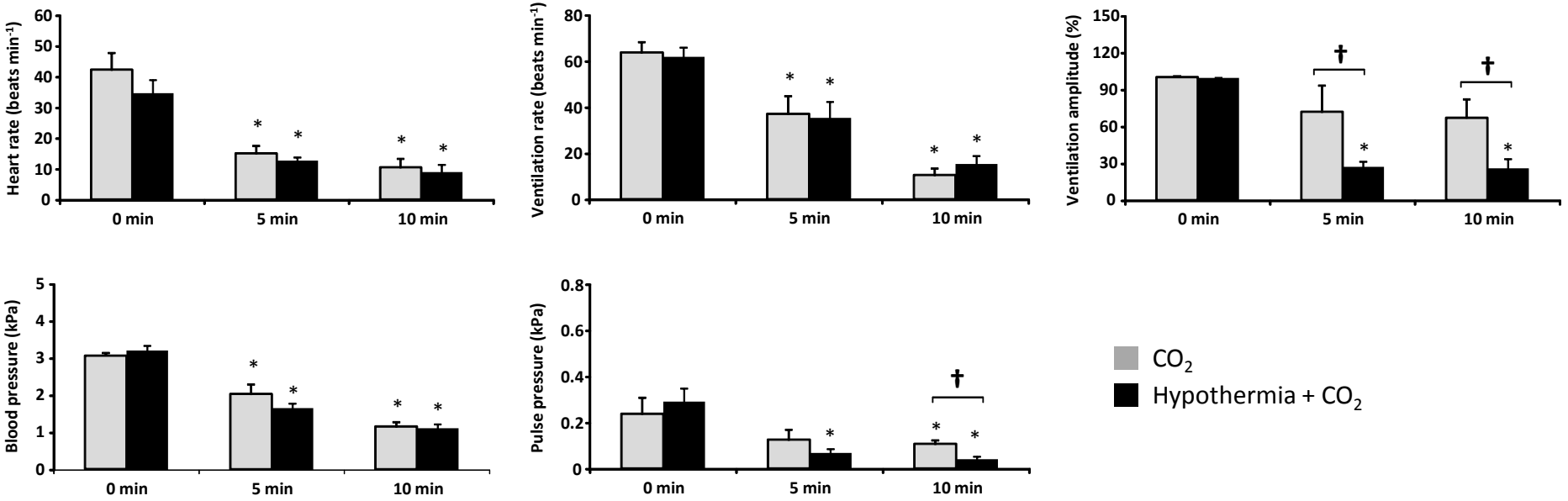

Heart rate (beats min-1)
60
50
40
30
20
10
0
*
*
*
0 min
5 min
30 min
+20 min

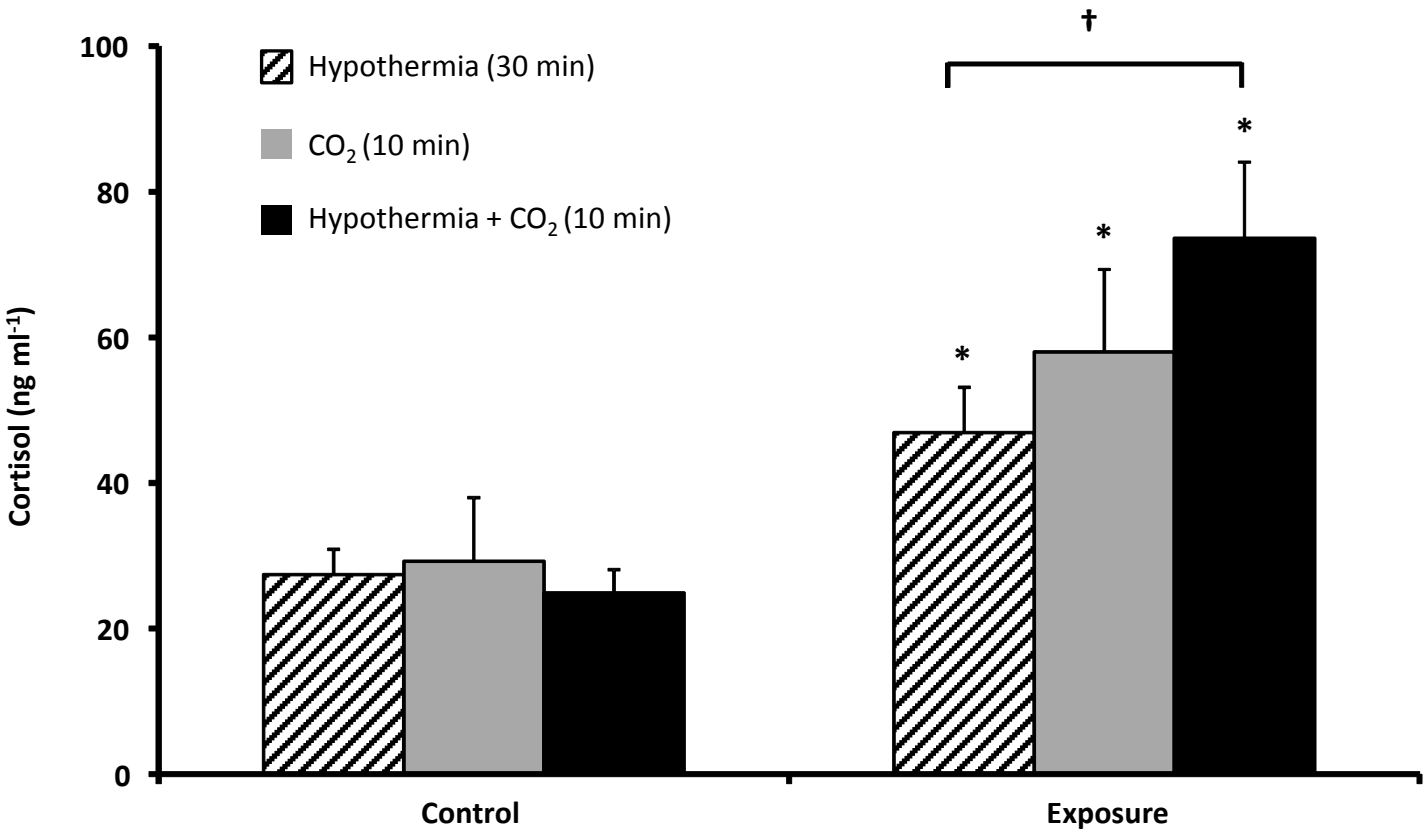

Ventilation rate (beats min-1)
80
60
40
20
0
*
*
*
0 min
5 min
30 min
+20 min


Ventilation amplitude (%)
150
120
90
60
30
0
0 min
5 min
30 min
+20 min


Blood pressure (kPa)
5
4
3
2
1
0
*
*
*
0 min
5 min
30 min
+20 min


Pulse pressure (kPa)
0.8
0.6
0.4
0.2
0
0 min
5 min
30 min
+20 min


Heart rate (beats min-1)
60
50
40
30
20
10
0
0 min
5 min
10 min
Ventilation rate (beats min-1)
80
60
40
20
0
Ventilation amplitude (%)
150
120
90
60
30
Blood pressure (kPa)
5
4
3
2
1
Pulse pressure (kPa)
0.8
0.6
0.4
0.2
CO2
Hypothermia + CO2


Hypothermia (30 min)
CO2 (10 min)
Hypothermia + CO2 (10 min)
Cortisol (ng ml-1)
100
80
60
40
20
0
Control
Exposure
†
*
*
*