Effect of confinement and starvation on stress parameters in the American lobster (Homarus americanus)

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Abstract

The American lobster (Homarus americanus) is one of the most important crustacean resources in North America. In Italy and Europe, this fishery product is available in non-feeding conditions. The current Italian legislation does not oblige retail operators to sell live animals. However, in the guidelines of the Italian law it is explicitly suggested to sell live animals in order to improve their qualitative characteristics. Furthermore, for many consumers, to buy live animals is perceived as a good and fair practice. At the retail level, lobsters are kept in temperature controlled aquaria at very low temperatures (8°C) in order to reduce metabolic processes and cannibalism. According to some studies, transportation and stocking conditions may cause stress to animals and reduce their welfare (Beard and McGregor, 2004; Danford et al., 2001; Lorenzon et al., 2000). Rapid environmental changes may cause stressful conditions for crustaceans, increasing the vulnerability to bacteria and reducing the immune response (Mercier et al., 2006). Jones (2009) showed a significant correlation between fishing method and survival rate of lobsters during the stocking phase. The physiological response of lobsters to external stressors mobilizes energy substrates (Zhou et al., 2011). Hyperglycemia and release of lactate in the hemolymph are the typical crustacean responses (Aparicio-Simon et al., 2010). Several studies have shown an increase in glucose concentrations in the hemolymph under stressful conditions such as handling (Bergmann et al., 2001), changes in salinity (Spaargen and Haefiner, 1987), diseases and pollutants (Lorenzon et al., 2004), temperature changes during transportation (Lorenzon et al., 2007), and the confinement for long periods with other individuals (Rügge et al., 2006). The release of crustacean hyperglycemic hormone (CHH) is the most important mediating neuromodulatory mechanism involved in the stress response of crustaceans and it is modulated by different neuromodulators such as catecholamines, dopamines, norepinephrines and epinephrines (Aparicio-Simon et al., 2010). Other variables involved in the stress response are the concentration of the total protein in the hemolymph (Jones, 2009; Lorenzon et al., 2007) and the total haemocyte count (THC) (Lorenzon et al., 2007). The aim of this study was to evaluate the effect of confinement for one month and feed deprivation on some properties of the hemolymph and muscle degradation pattern of American lobsters.

Introduction

Lobster fishery (Homarus americanus) is one of the most important economic activities in United States and Canada (mainly Northeast America and Atlantic Canada). In North America, total landings have substantially increased in the last decades (Phillips et al., 2013), while, on the other hand, total number of lobster catches in the Mediterranean countries (Italy, Turkey, Croatia, etc.) has been very low (close to zero) (Phillips et al., 2013). Lobsters are traditionally marketed live and kept in recirculating aquaria for several weeks in non-feeding conditions. The current Italian legislation does not oblige retail operators to sale live animals. However, in the guidelines of the Italian law it is explicitly suggested to sell live animals in order to improve their qualitative characteristics (Candotti, 2007). Furthermore, for many consumers, to buy live animals is perceived as a good and fair practice. At the retail level, lobsters are kept in temperature controlled aquaria at very low temperatures (8°C) in order to reduce metabolic processes and cannibalism. According to some studies, transportation and stocking conditions may cause stress to animals and reduce their welfare (Beard and McGregor, 2004; Danford et al., 2001; Lorenzon et al., 2000). Rapid environmental changes may cause stressful conditions for crustaceans, increasing the vulnerability to bacteria and reducing the immune response (Mercier et al., 2006).

Materials and methods

Animals and experimental design

Homarus americanus specimens were caught in the North Atlantic waters, specifically in the area of fisheries 4X, off the coast of Nova Scotia (Canada), in the period between the 31st December 2012 and 10th February 2013 and exported by the Newell Lobster factory (Yarmouth, Canada) to Groton (CT, USA). Lobsters were dry transported by airplane to Italy, departing from the airport J.F. Kennedy, New York, in April 2013. After their arrival in Venice, they were transported to Chioggia to the CAM srl factory. On 16th April, lobsters were dry transported by airplane to Italy, departing from the airport J.F. Kennedy, New York, in April 2013. After their arrival in Venice, they were transported to Chioggia to the CAM srl factory. On 16th April, lobsters were picked up and taken to Udine. The map of the route is shown in Figure 1. The research study was performed in the aquaria of the Food Science Department, University of Udine, Italy for one month. Thirty-six adult Homarus americanus males (initial weight of approximately 530 g) were reared in four temperature controlled and recirculating aquaculture tanks (0.56 m² each, 10% water volume replacement per day) using natural marine water. Aeration diffusers were provided within each tank. Lobsters were kept without food for four
weeks. The stocking density of lobsters was of 16/m² (similar to the average values used in Italy in the retail markets). Claws of lobsters were tied to avoid injuries and no shelters were provided in the tanks. During the experiment, water parameters (temperature, O₂, pH, N-NH₄, N-NO₂, N-NO₃, salinity) were analyzed (APHA, 1980) daily. A daily photoperiod of 12 h was maintained all throughout the experiment. Light was supplied by a 40 W white fluorescent tube (350 lux at the water surface). Tanks were covered on the top to reduce light intensity. At the arrival, all lobsters were acclimated in separate tanks for 48 h in the aquarium. Light was supplied by a 40 W white fluorescent tube (350 lux at the water surface). During the experiment, the total mean body weights of lobsters increased slightly compared to the means (SPSS, 2007). All the other growth and welfare parameters were analyzed by means of the Student’s test (SPSS, 2007). Levene’s test was used to assess the homogeneity of variances.

**Results and discussion**

**Water quality analysis**

Water quality parameters measured during the experiment are presented in Table 1. Results of water analysis showed no significant differences in temperature, pH, ammonia and nitrate (with the only exception of nitrite concentrations at the end of the third week: P<0.05; 16 df; standard error of variance (SE)=0.45) concentrations in the closed recirculating system.

**Growth parameters**

During the experiment, the total mean body weights of lobsters increased slightly compared to the means (SPSS, 2007). All the other growth and welfare parameters were analyzed by means of the Student’s test (SPSS, 2007). Levene’s test was used to assess the homogeneity of variances.

**Determination of hemolymph parameters**

Hemolymph samples were withdrawn from the pericardial sinus of each animal using a 2.5-mL syringe at the beginning of the experiment and every week. Samples were tested for glycemia, total protein content and THC. Each sample was centrifuged for 2 min at 5000 rpm and the supernatant was transferred and kept at -20°C until the analyses. Glucose content was measured from the supernatant using One touch® II meter (Lifescan, Milpitas, CA, USA) and a commercial kit test. The concentration of total protein was determined by means of the screen point method (Hospitex Diagnostics srl, Osmannoro-Sesto Fiorentino, Italy). The number of haemocytes were determined using 400 µL of hemolymph fixed with 1600 µL of 1% formalin in phosphate buffered saline and stored at +4°C.

**Statistical analysis**

All the statistical analysis were performed using the SPSS 14 package for windows. Water quality data were subjected to ANOVA and Duncan’s multiple range test was used to compare the means (SPSS, 2007). All the other growth and welfare parameters were analyzed by means of the Student’s test (SPSS, 2007). Levene’s test was used to assess the homogeneity of variances.

**Table 1. Changes in water quality parameters during the trial.**

| Variables          | Storage time, weeks | SE (17 df) |
|--------------------|---------------------|------------|
|                    | T₀    | 1ʰ     | 2ʰ     | 3ʰ     | 4ʰ     |
| Temperature, °C    | 8.5   | 8.3    | 7.8    | 8.1    | 7.5    | 0.44    |
| pH                 | 7.5   | 7.6    | 7.6    | 7.8    | 7.8    | 7.7     | 0.007   |
| NH₄-N, mg/L        | 1.6   | 1.7    | 1.3    | 0.6    | 1.1    | 0.033   |
| NO₂-N, mg/L        | <5    | <5     | <5     | <5     | <5     | <5      | <5      |<5      |
| NO₃-N, mg/L        | <5    | <5     | <5     | <5     | <5     | <5      |<5      |
| O₂, mg/L           | 6.4   | 6.7    | 7.3    | 7.1    | 6.8    | 1.70    |
| O₂ % sat           | 72.0  | 73.8   | 81.3   | 83.3   | 77.4   | 215.4   |
| Salinity, %        | 33.0  | 33.0   | 33.0   | 33.0   | 33.1   | 0.25    |

SE, standard error of variance; df, degree of freedom. *P<0.05; **P<0.01. Different letters in the same row denote significant differences among treatments.
pared to the initial values (Table 2). This result was probably due to the combined effects of water loss during the transportation phase (exposure to air) and water intake of animals during the confinement period. Although the change in live body weights was not statistically significant, the general trend was a slight constant increase of weights during the experiment.

**Hemolymph parameters**

Glucose, total protein concentrations and THC in the hemolymph of *H. americanus* during the experiment are shown in Figures 2 to 4, respectively. After one month of confinement, mean glucose, protein and THC levels in the hemolymph of *H. americanus* were similar (P>0.05) to the initial values. Although the overall mean differences of glucose and total protein concentrations were not significant at the statistical analysis, during the experiment the highest values were observed after two weeks, while the lowest value of THC after one week (P<0.01; 40 df; SE=268529; 1st week vs other treatments). This pattern seems to indicate a temporary situation of stress for animals, showing both the mobilization of energy (glucose) and total protein (presumably due to the synthesis of hemocyanin). The improvement of glucose concentrations in the 3rd and 4th week could indicate a lower organic load of the biological filter and/or that lobsters were no longer capable to deplete the glycogen stores. In the literature, some threshold values are reported indicating stress in crustaceans: glucose>15 mg/dL, total protein>6 g/dL, and THC<1500 mm²/10,000 (Lorenzon *et al.*, 2004, 2007). In many species, it was observed that the first response to stress is the mobilization of glucose. The immune system of crustaceans is mainly represented by haemocytes, cellular component of the hemolymph, divided in three types: hyaline cells, involved in phagocytosis, granular and semigranular hemocytes. Granules contain elements of the prophenoloxidase system, an enzymatic cascade system responsible for the production of melanin, involved in the mechanisms of defense (Söderhäll and Smith, 1986; Smith and Söderhäll, 1991). In the literature, the role of hemolymphatic total protein in crustaceans is well documented (Brouwer *et al.*, 2004). This metabolite can therefore be used as indicator of the environmental stress (Brouwer *et al.*, 2004). Many authors agree that the key component is the hemocyanine (Hc), the respiratory pigment, representing 80 to 90% of the total protein in the hemolymph of crustaceans (Chen and Cheng, 1995; Watt *et al.*, 1999; Chausson *et al.*, 2004). Although the physiological stress induces also the mobilization of other proteins, including the metallothionein and the stress proteins (Cimino *et al.*, 2002; Ravaux *et al.*, 2007). Hcs are widely used for monitoring the welfare condition of crustaceans. Hemocianines are oligomeric proteins formed by the aggregation of subunits whose molecular weights are between 65 and

| Table 2. Variation of body weights of lobsters during the experiment compared to the initial value. |
|---------------------------------------------------------------|
| **Variable** | **Tank** | **T₀** | **End of the trial (weeks)** |
|----------------|---------|--------|-----------------------------|
| Body weight, g | 1       | 534±26.0 | 540±24.7 (1st) |
|                | 2       | 546±38.0 | 553±39.7 (2nd) |
|                | 3       | 526±43.6 | 541±39.9 (3rd) |
|                | 4       | 570±39.9 | 576±39.2 (4th) |

Figure 2. Variation of glucose concentrations in hemolymph of *H. americanus* during the experiment. Values are expressed as means±standard error.

Figure 3. Variation of protein concentrations in hemolymph of *H. americanus* during the experiment. Values are expressed as means±standard error.
90 kDa. As pointed out by Dolashka-Angelova et al. (2001), most of Hcs are glycoproteins, wherein the carbohydrate component varies from species to species. It has also been observed that the number of subunits constituting the Hcs has an interspecific variability (Giomi and Beltramini, 2007). With respect to this, Hodgson and Spicer (2001), analyzing the electrophoretic pattern of hemolymph, found that Hcs of different species of decapods have a number of subunits between six and ten. The prolonged exposure of animals to stress conditions may determine the appearance of undesirable effects such as the tissue oxidation with effects on the quality of the final product (Hermes-Lima and Ginger-Savin, 2002). Several antioxidant enzymes such as the superoxide dismutase, catalase and glutathione-S-transferase have been identified as a possible key elements in crustacean defense systems (against the free radicals) (Halliwell and Gutteridge, 2007). These enzymes remove or transform the free radicals in less toxic metabolites (Halliwell and Gutteridge, 2007). When the production of antioxidants is not sufficient to contrast the production of free radicals, the result is the oxidative stress, i.e. the increase of oxidative damage at the cell level (Almeida et al., 2005). These damages are the oxidation of DNA, lipids and proteins that can alter the normal cellular functions (Halliwell and Gutteridge, 2007). In a study on crab *Paralomis granulosa*, the antioxidative enzyme activity was used as an indicator of the cell damage caused by different types of air exposure (Romero et al., 2007). In another study, Romero et al. (2011) examined the antioxidative activity in the same crab *Paralomis granulosa* after the exposure to air for 6 h at 7°C and re-submersion in the water. The highest concentrations of antioxidant enzymes were detected in the gills after the water re-submersion. In the same research, an additional experiment was carried out to test whether the stress due to air exposure (for increasing times), affected the flavor of the final product. Despite observing some differences in the flavor of the meat, it was not possible to accurately distinguish the different treatments.

**Proximate analysis**

The proximate composition of muscle at the end of the experiment is reported in Figure 5.

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**Figure 4.** Variation of total haemocyte count concentrations in hemolymph of *H. americanus* during the experiment. Values are expressed as means±standard error.

**Figure 5.** Proximate muscle composition of *H. americanus* during the trial.

**Figure 6.** Time course with anti-actin detection.

**Figure 7.** Time course of total proteins in Coomassie staining.

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Results of the proximate analysis showed no significant differences in the concentrations of crude protein (P>0.05; 40 df; SE=0.72), total lipids (P>0.05; 40 df; SE=0.01) and ash (P>0.05; 40 df; SE=0.01) in the muscle of *H. americanus* at the end of the experiment. Some studies reported a change in the chemical composition of the muscle tissue as a result of different stocking conditions (Barrente et al., 2009; Fang et al., 1992). For instance, Fang et al. (1992) observed a variation in the concentration and composition of free amino acids (FAA) in crustaceans according to the salinity of the water. The pool and the concentration of the FAA in the muscle may influence the quality and flavour of the final product (Wang et al., 2004; Zhou et al., 2011).

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis**

At the end of the experiment, the SDS-PAGE analysis revealed a marked degradation of the myofibrillar proteins. The degradation of the muscle protein was analysed through the identification of actin and myosin extracts and their possible products of proteolysis over time (Figures 6 and 7). A number of fragments, possibly from myosin, was evident in the range between 50 and 220 kDa between the time 3 and 7. A proteolytic product of 37 kDa, recognized by the anti-actin antibody, was also detected. The formation of new bands is probably due to the protein degradation under the fasting process. The Western Blot method against the C-terminal actin was also helpful in identifying these proteolytic fragments derived from the native actin. In vitro experiments have clearly shown that the myosin of lobsters is particularly susceptible to proteolytic attacks (Siemannowski et al., 1980). In the present experiment, after 28 days, it was evident a new band of 37 kDa, identified as a C-terminal fragment of the actin protein. These results suggest that, during the stocking phase, the muscle of lobsters undergoes degradation of both thick and thin filaments. Such degradation may be due to stress induced by fasting, which is well known to induce muscle degradation both in mammals and fish (Iliyan and Forsberg, 1992; Preziosa et al., 2013). The muscle of lobsters contains different isoforms of calpain (Gornik et al., 2010) and ubiquitin/proteasome, which mediate the turnover of myofibrillar proteins. Salem et al. (2005) observed an increase of mRNA of Capn1 and Capn2 after a fasting period of 35 days in rainbow trout. Other systems can also be activated by non-feeding conditions resulting in the degradation of the fibrillar proteins (Mykles, 1997).

**Conclusions**

Crustaceans are able to display a wide range of adaptation strategies to survive and grow. The overall knowledge of these complex biological systems could be of importance in order to identify useful welfare indicators. In this study, some hemolympathic variables and muscle degradation analysis (SDS-PAGE and Western blotting) resulted as sensitive indicators of stress in *H. americanus*. Furthermore, in the present research no statistical effects of starvation and storage of *H. americanus* were observed on body chemical composition. Further research work will be needed in the near future on several quality parameters related to freshness, such as sensory evaluations and k value. Starting from these results, several practical guidelines may be applied by retail operators in order to develop an optimal stock management system of *H. americanus*.

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