

INFLUENCE OF SAMPLING, STORAGE, PROCESSING AND OPTIMAL EXPERIMENTAL CONDITIONS ON ADENYLATE ENERGY CHARGE IN PENAEID SHRIMP

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Abstract - Adenylate energy charge (AEC) has been used as a practical index of the physiological status and health in several disciplines, such as ecotoxicology and aquaculture. This study standardizes several procedures for AEC determination in penaeid shrimp that are very sensitive to sampling. We concluded that shrimp can be frozen in liquid nitrogen and then stored at -76°C for up to two years for further analysis, or freshly dissected and immediately homogenized in acid. Other cooling procedures, such as immersion in cold water or placing shrimp on ice for 15 min resulted in 50% and 73% decreases in ATP levels, and 9-fold and 10-fold increases in IMP levels, respectively. Optimal values of AEC (0.9) were obtained in shrimp recently transferred from ponds to indoor conditions, but decreased to 0.77 after one month in indoor tanks when stocked at high densities; the AEC re-established to 0.85 when the shrimps were transferred to optimal conditions (lower density and dark tanks). While the levels of arginine phosphate followed the same pattern, its levels did not fully re-establish. Comparison of different devices for sample homogenization indicated that a cryogenic ball mill mixer is the more suitable procedure.

Key words: Adenylic energy charge, ATP, IMP, *Litopenaeus* (= *Penaeus*) *vannamei*, methods; stress

INTRODUCTION

Adenylate energy charge (AEC) was defined by Atkinson and Walton (1967) as the relationship between energy-charged nucleotides to total nucleotides by the equation $(\text{ATP} + \frac{1}{2}\text{ADP}) / (\text{ATP} + \text{ADP} + \text{AMP})$. Although the AEC is supposed to be tightly regulated, after significant perturbations it decreases and can reach lower values in invertebrates as compared to vertebrates (Raffin and Thébault, 1996). AEC is also considered an indicator of “well-being” of an organism (Ivanovici, 1980a), as a diagnostic tool about an organism’s response to sublethal changes in the environment (Ivanovici, 1980b), and as the capacity of an organism’s response to adverse conditions, as for example sublethal stress (Marazza et al.,

1996). AEC was estimated for multiple applications in aquatic invertebrates, such as examination of the effects of environmental perturbations on the physiological status. Analysis of AEC and phosphagens is essential to understand the metabolic response to exhaustive muscular work or hypoxia (Onnen and Zebe, 1983; Gäde, 1984; Thébault et al., 1994; Morris and Adamczewska, 2002; Abe et al., 2007; Gornik et al., 2010).

One of the most common applications of AEC and related variables is in the ecotoxicological context where it is used to test for xenobiotic effects on metabolic physiology of different mollusk and crustacean species (Marazza et al., 1996; Thébault et al., 1996; Harris and Santos, 2000; Thébault et al., 2000;

Morris et al., 2005; Le Moullac et al., 2008). Some applications related to aquaculture or fisheries were also tested on commercial species of mollusks and crustaceans. For the Pacific oyster *Crassostrea gigas*, AEC has been proposed as a trophic index for intensive aquaculture management (Moal et al., 1991) and as an indicator of reproductive effort, depending on environmental constraints (Moal et al., 1987). It was used to examine the energy status when analyzing the phenomena of oyster mortality in summer in France (Samain and McCombie, 2007). By assessing AEC, it was possible to establish the best transport method for great scallop, *Pecten maximus*, juveniles, even if no mortality occurred (Maguire et al., 1999), and to assess the impact of capture by dredging (Maguire et al., 2002). For crustaceans, variations in AEC were investigated in an eco-physiological context in response to the effects of temperature on growth for the oriental river prawn *Macrobrachium nipponense* (Wang et al., 2006) and in relation to the optimal pH range for the fleshy prawn *Fenneropenaeus* (= *Penaeus*) *chinensis* (Wang et al., 2002). The AEC also served as an index of physiological viability for the giant tiger prawn *Penaeus monodon*, and kuruma prawn *Marsupenaeus* (= *Penaeus*) *japonicus*, in response to transport out of water in sawdust (Paterson, 1993). To our knowledge, except for these latter studies and the influence of hypoxia on *M. japonicus* (Abe et al., 2007), no other study about the AEC of live penaeid shrimp exists. AEC can be an important tool for understanding physiological processes, and it can serve as a practical index of health and the physiological status under different natural or domesticated conditions.

Despite the wide use of AEC in comparative physiology studies of marine invertebrates mentioned above, some methodological questions regarding the best way to sample and process remain unanswered, including the stability of samples during long-term storage. The majority of such studies of mollusks and crustaceans were performed by obtaining samples in liquid nitrogen following different particular variants, such as direct immersion of living shrimp (Paterson, 1993; Marazza et al., 1996), shrimp tail (Wang et al., 2002; Abe et al., 2007) or

whole oysters after quick separation from shell (Moal et al., 1991; Le Moullac et al., 2008), use of particular accessories, such as “freeze clamp” tongs for muscle of crayfish (Morris et al., 2005), or quick dissection of different tissues from scallops (Maguire et al., 1999), decapod crustaceans (Giesy et al., 1981; Albalat et al., 2009; Gornik et al., 2010), or fish (Mendes et al., 2001) before freezing. The procedures using freezing during sampling has the advantage of stopping further reaction during tissue sampling. Analysis of mice brains indicates that certain changes in the concentration of nucleotides are induced following the immersion of whole mice in liquid nitrogen, probably by the induction of arterial hypoxia before the tissue was frozen (Pontén et al., 1973). In a recent study, the immersion of slices of rat brain in liquid nitrogen resulted in lower values of AEC compared to slices of rat brain perfused with a cold saline solution, although these differences were attributed to the processing of powder obtained by freezing with liquid nitrogen due to the reactivation of enzymes during melting before proteins are precipitated (zur Nedden et al., 2009). Therefore, using liquid nitrogen should be validated for specific samples and further mechanical processing should be evaluated.

The processing method varies in different studies, but for harder tissues, such as muscle, it generally involves grinding frozen tissue under cryogenic conditions in a ceramic mortar (Wang et al., 1994; Morris et al., 2005), steel grinders (Ivanovici, 1980b; Giesy et al., 1981), and particularly with a ball mill mixer, e.g. “Dangoumeau” (Moal et al., 1991; Le Moullac et al., 2008). In some studies, the tissue is directly homogenized in a cold acid solution with the use of different mechanical devices, such as a Waring blender (Paterson, 1993), Polytron (Mendes et al., 2001) or Ultra-Turrax homogenizers (Raffin and Thébault, 1991; Gornik et al., 2008). However, practically no studies have systematically compared different methods of sample processing on AEC. A preliminary comparison of an individual sample in which Potter processing resulted in lower stability of the neutralized extract, compared with previous grinding with a Dangoumeau mixer (Moal et al., 1989). For fish (Atlantic salmon and rainbow trout),

the maceration of muscle on ice resulted in much lower concentrations of ATP than in samples ground into a fine powder in a mortar cooled with liquid nitrogen or on dry ice (Thomas et al., 2000).

Regarding storage of samples, two issues could have important practical implications: long-term storage of partially processed samples (e.g., crude protein-free extracts or neutralized extracts ready to be injected into an HPLC) or unprocessed tissues or whole animals frozen at -70°C . In the first case, Moal et al., (1989) found no variation of adenylic nucleotides in neutralized extracts stored at -20°C over four months, but no studies have analyzed the first issue.

Finally, the baseline conditions under which organisms are sampled and the way they are captured should be of primary concern to avoid stress that shifts the energy balance to an uncontrolled variation. Therefore, optimal conditions of stocking and capture of organisms under wild, farmed, or experimental conditions should be established to obtain baseline or control values of adenylic nucleotides and the resulting AEC.

This study analyzes the concentration of adenylates and AEC in relation to different procedures used to obtain shrimps from tanks and samples from shrimp, long-term storage, homogenizing of samples.

MATERIALS AND METHODS

Animals

Depending on the particular experiment, juvenile whiteleg shrimp *Litopenaeus* (= *Penaeus*) *vannamei* were obtained from outdoor concrete tanks (1.4 m^2) or tidal ponds ($8\,000\text{ m}^2$) at the CIBNOR facilities. In both cases, general conditions were stocking density ~ 40 shrimp/ m^2 , temperature between 26°C and 28°C , oxygen $>4\text{ mg L}^{-1}$, salinity between 36 and 39, pH 7.8, ammonia levels below 1 mg L^{-1} , and natural photoperiod. The shrimps were fed a commercial diet containing 35% protein (PIASA, La Paz, B.C.S., Mex-

ico) at a ratio of 2% in ponds and 5% or less in tanks, depending on apparent consumption. In tanks, water was exchanged at a rate of 50% per day to remove feces and food that was not ingested. Shrimp weight was between 15 g and 20 g. Shrimp from tanks were sampled *in situ*. When shrimp from pond were used for particular experiments, they were transferred and acclimated to different indoor tanks, depending on the experimental group, as specified below. General conditions in indoor tanks were similar to those of outdoor tanks described above.

Experiments and sample processing

Sampling: influence of liquid nitrogen freezing versus quick dissection at room temperature

Shrimps were obtained from outdoor tanks; whole shrimp was either immersed in liquid nitrogen ($n = 4$), or killed by cutting the tail between the first abdominal segment and the cephalothorax ($n = 4$). A piece of abdominal muscle was quickly dissected and separated from the exoskeleton on a cold plate, weighed ($\sim 150\text{ mg}$), and immersed in 1.5 ml cold 0.5 M trichloroacetic acid (TCA). Dissection of a fresh sample was completed within 30 to 40 s. For frozen shrimp, the same procedure was applied and samples were not defrosted until immersion in TCA. Samples were immediately homogenized with a mechanical homogenizer (model Tempest IQ2, The VirTis Company, Gardiner, NY).

Sampling: influence of different cooling method

Availability of liquid nitrogen or dry ice at the sampling sites for shrimp (farms, estuaries or open sea) depended on planned protocols with the transport of containers with liquid nitrogen or dry ice from research facilities. Therefore, immediate sampling at a given time to check for particular conditions for ecotoxicological or aquaculture purposes was not possible. The possibility of using traditional cooling methods for shrimp harvest and fishing was then investigated. Shrimp were obtained from outdoor concrete tanks and sampled according to one of the following. (1) Immersion in liquid nitrogen

following massive capture ($n = 11$). Shrimps were individually immersed in liquid nitrogen; however, these shrimp were first exposed to air for between 10 s and 3 min because they were captured in one batch and remained in the net until frozen. This procedure (massive capture with a short exposure to air) happens in normal sampling of shrimp in ponds or from a natural environment and therefore it was interesting to check if the samples obtained in this way were suitable for AEC analysis. (2) Immediate immersion in liquid nitrogen following individual capture ($n = 10$). Shrimps were individually captured from tanks and immediately immersed in liquid nitrogen. For both groups, shrimps were extracted from the liquid nitrogen tank and stored at -76°C until processing and analysis. (3) Harvest of shrimp in cold water ($n = 8$). Shrimps were captured in bulk with a net and immediately transferred to a container of seawater cooled to 7°C with ice, left for 15 min in this condition, and then frozen at -76°C until sample processing and analysis. Within 15 min, no shrimp responded when handled and were presumed dead. (4) Harvest of shrimps on ice ($n = 9$). Shrimps were captured in bulk with a net and immediately placed in a container of ice, left for 15 min in this condition, and then frozen at -76°C until sample processing and analysis. During the 15 min, shrimp were cooled, but also exposed to air. In fact, at the end of this period, some shrimp still presented the typical escape response when handled.

Long-term storage of shrimp at -76°C

Shrimps from outdoor concrete tanks were individually captured, immersed in liquid nitrogen, and individually packed in polyethylene bags. After sampling, shrimps were frozen at -76°C and processed after a variable period of time ranging from 7 to 690 days. These shrimps were grouped according to the duration of storage as follows: 1 week, 3 weeks, 3 months, 6 months, and 23 months ($n = 5$ for each group).

Baseline optimal condition of shrimp for sample collection

Previous results indicate that lower values of AEC

are obtained in shrimp housed long term in indoor tanks; on the other hand, controlled experiments could not be conducted on shrimps raised in outdoor tanks or ponds. Therefore, the present experiment compared the following conditions in specimens that fasted for 12 h. (1) Shrimps obtained from ponds and transferred to 30 l individual indoor tanks and sampled the next day ($n = 16$). (2) Shrimps obtained from ponds, stocked in small clear tanks (500 l) under indoor conditions for one month at a density of 70 shrimps/ m^2 , and then transferred to 30 l individual indoor tanks and sampled the next day ($n = 5$). (3) Shrimps obtained from ponds, stocked for one month in small tanks (500 L), then transferred to large 5 000 l dark tanks, also under indoor conditions for another one month at a density of 20 shrimps/ m^2 , and then transferred to 30 l tanks until the next day for individual sampling, as in the previous two groups ($n = 22$).

All shrimps were individually captured and immediately immersed in liquid nitrogen, stored at -76°C , and processed within one month.

Tissue processing: different methods of homogenization

Shrimps from ponds were transferred to 30 l individual indoor tanks, sampled the next day by immersion in liquid nitrogen, stored at -76°C , and processed within one month. Three different instruments were used to compare quality of homogenization of a sample of the abdominal muscle. (1) A sample of muscle (~ 150 mg) was immersed in 1.5 ml cold TCA, cut into small pieces and disrupted in a bead beater (model FastPrep24, MP Biomedicals, Irvine, CA) for 40 s and 40 cycles ($n = 6$). (2) A sample of muscle was treated as in the second group, but the tissue was directly homogenized with a mechanical homogenizer (model Tempest IQ2, VirTis) at 10 000 rpm for 30 s in ice ($n = 6$). (3) Muscle was pulverized under cryogenic conditions (liquid nitrogen) with a ball mill mixer (MM400, Retsch, Haan, Germany) for 2 min at a frequency of 25/s. The frozen powder (~ 150 mg) was homogenized in 1.5 ml cold TCA with a rotor/stator mechanical homogenizer (model Tempest

IQ2, The VirsTis Company) in ice ($n = 6$).

The crude extract obtained from all groups was centrifuged at 3 000 g for 10 min at 4°C, and the resulting supernatant (free of proteins) was used for extraction of nucleotides and arginine phosphate.

Extraction and analysis of nucleotides and arginine phosphate

Extraction and analysis of nucleotides was performed as described by Moal et al. (1989), with some modifications. For neutralization of the acid extract, dichloromethane was used instead of amine-freon, according to a previous standardization done on oyster tissue in which no differences were obtained between both solvents (Moal, pers. comm.). For this purpose, 500 μL of the acid supernatant was mixed with 1 300 μL of a mixture of dichloromethane/trioctylamine (5:1 v/v), vigorously mixed with a vortex for 1 min; pH ≥ 6 was checked with pH paper. In a few cases, when the pH was still slightly acidic, a few drops of the dichloromethane/trioctylamine mixture were added to the extract, mixed, and the pH was checked again. A two-phase system was obtained in which the upper aqueous phase contained nucleotides at a neutral pH.

Nucleotides were separated by ion pairing reverse phase high-performance liquid chromatography (model 1100, Agilent Technologies, Santa Clara, CA) with an octadecylsilane (ODS) C18 column (Hyper Clone 150 mm long, 4.6 mm width, 3 μm particle size diameter, Phenomenex, Torrance, CA), with a security guard cartridge C18 (40 mm long, 3.0 mm width, Phenomenex). Separation of nucleotides was performed under isocratic conditions, using a mobile phase of 0.15 M NaH_2PO_4 buffer, 3 mM tetrabutylammonium as the ion-pairing agent, 8% methanol, adjusted to pH 6.0 with 0.1 N NaOH. The chromatograph was operated at a 0.8 ml/min flow rate. Under these conditions, the separation of nucleotides was complete in less than 20 min (Fig. 1). The nucleotides were detected at 254 nm (Agilent detector coupled to HPLC system). Identification and concentration calculations of nucleotides were performed with

standards of ATP, ADP, AMP, GTP, GDP and IMP (all from Sigma, St. Louis, MO).

Arginine phosphate (ArgP) was also analyzed in samples of some experiments from the same extract used for nucleotides analysis. Analysis of ArgP was performed by HPLC, according to Viant et al. (2001), using a reverse-phase SphereClone NH_2 column (250 mm length, 4.6 mm width, 5 μm particle size diameter, Phenomenex) fitted with an NH_2 security cartridge (40 mm long, 3 mm wide, Phenomenex). Identification and concentration calculations of ArgP were performed with a standard of purified ArgP (Santa Cruz Biotechnology, Santa Cruz, CA).

All solvents used for HPLC analysis were prepared using HPLC grade commercial reagents and de-ionized water, and then filtered using a 0.45 μm nylon membrane.

Statistical analysis

Variables were checked for normality and homogeneity by the Shapiro-Wilk and Levene tests, respectively, and when one of these conditions was not fulfilled, the data were transformed and tested again. One-way ANOVA was followed by a Newman-Keuls test for mean comparisons. Spearman's correlation coefficients between AEC values and ArgP concentrations and the number of tail flips were calculated in experimental and baseline conditions. The correlation between AEC and the elapsed time from the beginning of the sampling was also calculated for the data obtained in the experiment on long-term storage. All analyses were performed using Statistica 8.0. (StatSoft, Tulsa, OK). The data are presented as means \pm SE; the differences are significant if $P < 0.05$.

RESULTS AND DISCUSSION

Extraction and analysis of nucleotides and arginine phosphate

Using dichloromethane instead of amine-freon to neutralize the acid extract was already tested in oyster tissues (Moal, pers comm.) or culture of neurons

(Hui et al. 2012), and further validated in our study for crustacean tissues. Solvents immiscible in water, such as the mixture amine-freon or dichloromethane with trioctylamine, when compared to classical procedures with salts, such as K_2CO_3 (Viant et al., 2001) or KOH (Gornik et al., 2010), have the added advantage of avoiding dilution of the sample. Moreover, during neutralization with K_2CO_3 , ATP may co-precipitate with potassium perchlorate (Moal et al., 1989). Finally, the neutralized extract with dichloromethane/trioctylamine was useful, not only for nucleotide analysis, but for arginine phosphate (ArgP) analysis by HPLC, in contrast to the common procedure to obtain the neutralized extract with K_2CO_3 (Viant et al., 2001).

Sampling: the influence of liquid nitrogen freezing versus quick dissection at room temperature

The concentration of adenylic nucleotides or the AEC was not significantly affected by the sampling procedure, although a trend of lower values of ATP and total adenylic nucleotides (TAN) was observed in freshly dissected shrimps (Table 1). In both groups, high values of AEC were observed (above 0.9), which is in agreement with values of AEC of 0.8–0.9 reported in unstressed animals under optimal conditions (Ivanovici, 1980b; Marazza et al., 1996; Gornik et al., 2010) and in samples not subjected to degradation (Gornik et al., 2008). Similarly, in the sea snail *Purpurus ebeninus*, Ivanovici (1980b) did not find any difference with time elapsed from the dissection to freezing of sample (1 to 4 min), although a non-significant decrease in AEC of 0.1 occurred between 2 and 4 min. In the same work, no significant correlation was obtained between AEC or TAN and the time taken for dissection within the typical range of dissection from 35 to 73 s.

Several studies assumed that immediate freezing by immersion in liquid nitrogen or by using freezer tongs was necessary to manipulate decapod crustaceans (Thébault et al., 1994; Marazza et al., 1996; Morris et al., 2005; Abe et al., 2007), although no comparison was done with freshly dissected samples. Evaluation was considered necessary because penaeid

shrimp reacts with a marked abdominal contraction when captured, and previous reports state that several decapod crustaceans have decreased AEC because of tail flipping (Onnen and Zebe, 1983; Gäde, 1984; Thébault et al., 1994; Morris and Adamczewska, 2002; Gornik et al., 2010). For the glass shrimp (*Palaeomonetes paludosus*), a lower value of AEC, due to higher levels of AMP were observed in one portion of freshly dissected muscle that was frozen within 50 to 60 s, compared to immediate freezing of whole crayfish or freezing of whole tail separated from the abdomen within 20 s (Giesy et al., 1981). Moreover, Paterson (1993) reports that whole kuruma prawns (*M. japonicus*) must be immersed in liquid nitrogen to avoid a substantial increase in AMP levels, in contrast to giant tiger prawn (*P. monodon*) for which the tail was first separated from the abdomen before freezing. From our results, we assume that there is no major problem for whiteleg shrimp (*L. vannamei*), not only to separate the tail from the cephalothorax before freezing, but also that a portion of tail muscle can be dissected from unfrozen shrimp, provided the procedure is rapid enough and samples are immediately homogenized in acid. No increase in AMP was observed and IMP was not detected in samples freshly dissected, further indicating that no substantial degradation of ATP was occurring. However, when many shrimp are sampled freezing in liquid nitrogen is highly recommended to avoid time-consuming processing of shrimp.

Lower AEC values were obtained in frozen brain slices from rats with respect to fresh slices (zur Nedden et al., 2009), although the authors suggested that ATP degradation was probably due to melting of the cryogenic powder during its manipulation and homogenization. Therefore, special care to manipulate the tissue powder always with liquid nitrogen should be taken, since the tissue powder melts more quickly than the entire tissue. In another study performed on mice brains, it was observed that the degradation of adenylic nucleotides and phosphagens occurred during the immersion of the entire body in liquid nitrogen because arterial hypoxia develops before the tissue is frozen (Pontén et al., 1973). In contrast to mammals, this seems unlikely to occur in aquatic in-

Table 1. Effect of liquid nitrogen freezing on the levels of several nucleotides and AEC in *Litopenaeus vannamei*

	Direct dissection	Frozen in liquid nitrogen
ATP	7.09 ± 0.51	7.93 ± 0.31
ADP	0.97 ± 0.38	0.94 ± 0.15
AMP	0.10 ± 0.08	0.05 ± 0.01
TAN	8.16 ± 0.87	8.92 ± 0.34
AEC	0.94 ± 0.02	0.94 ± 0.01

Concentration of all nucleotides is expressed as $\mu\text{moles/g}$ tissue (on a fresh weight basis).

TAN: Total adenyly nucleotides; Adenylate energy charge.

Data reported as means (\pm SE).

Table 2. Levels of different nucleotides, adenylate energy charge and arginine phosphate in *Litopenaeus vannamei* exposed to different cooling methods before sampling

	Group 1 (immediate immersion in liquid nitrogen)	Group 2 (delayed immersion in liquid nitrogen)	Group 3 (cooled in water at 5°C)	Group 4 (cooled on ice)
ATP	6.81 ± 0.36 ^b	3.57 ± 0.70 ^a	3.43 ± 0.54 ^a	1.83 ± 0.51 ^a
ADP	1.46 ± 0.14 ^a	2.94 ± 0.18 ^c	2.09 ± 0.23 ^b	2.33 ± 0.20 ^b
AMP	0.25 ± 0.07 ^a	1.64 ± 0.45 ^{bc}	0.95 ± 0.36 ^b	2.50 ± 0.46 ^c
TAN	8.52 ± 0.34 ^b	8.15 ± 0.35 ^b	6.46 ± 0.48 ^a	6.66 ± 0.38 ^a
AEC	0.88 ± 0.02 ^c	0.61 ± 0.06 ^b	0.68 ± 0.06 ^b	0.44 ± 0.06 ^a
IMP	0.04 ± 0.004 ^a	0.06 ± 0.01 ^a	0.35 ± 0.12 ^b	0.40 ± 0.15 ^b
ArgP	13.70 ± 3.44 ^b	2.92 ± 0.49 ^a	2.69 ± 0.74 ^a	2.79 ± 1.09 ^a

Means not sharing the same superscript are significantly different.

IMP = inosine monophosphate

ArgP = arginine phosphate ($\mu\text{mol/g}$ of tissue on a fresh weight basis)

See Table 1 for other specifications.

vertebrates due to their lower metabolism and higher tolerance in general to hypoxic conditions.

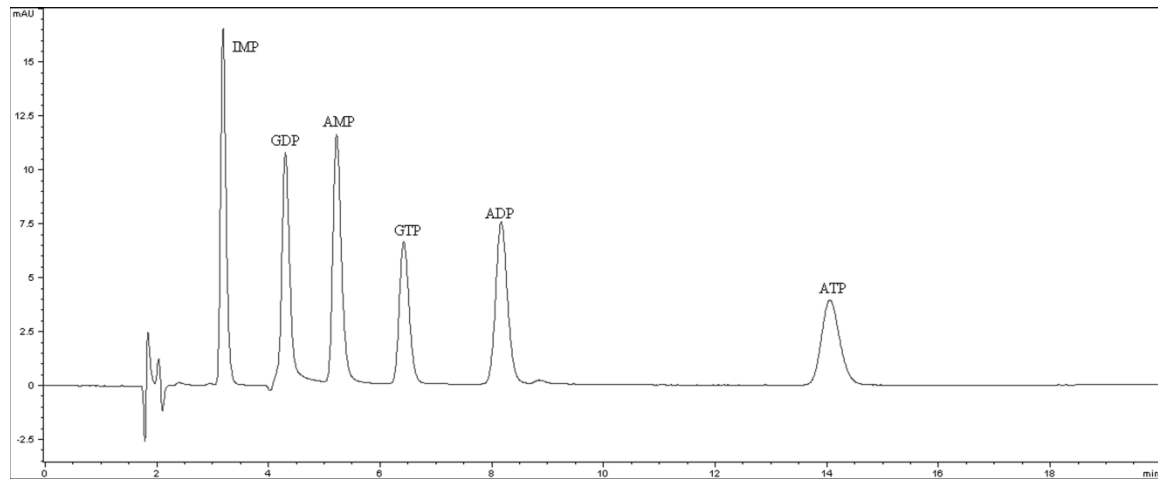
Sampling: the influence of different cooling methods – simulating a harvest or fishing process

Although the results of our first experiment indicate that fresh samples could be either immediately processed or frozen for further processing (this is further validated in the next experiment), these possibilities are not readily available at shrimp farms or natural environments, where immediate sampling could be of great interest to evaluate shrimp conditions at a given time. One possibility is to cool the

shrimps, as occurs during harvesting or fishing, and store them in -20°C to -30°C freezers for hours or a few days until they are transferred to the research facilities for nucleotide analysis. Therefore, the present experiment evaluated the influence of different cooling procedures on the concentration of nucleotides.

Significant differences in AEC, levels of individual adenyly nucleotides, particularly ATP, and ArgP were observed between shrimp that were individually frozen in liquid nitrogen compared to the other three groups (Table 2). Perhaps the most surprising result was the large decrease in ATP (and therefore in AEC) and ArgP from the massive sampling group

A)



B)

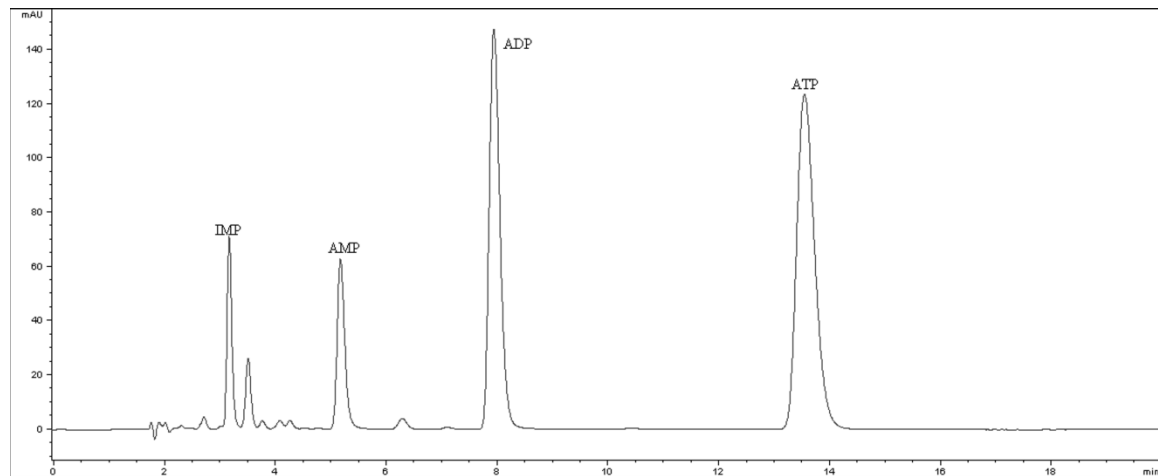
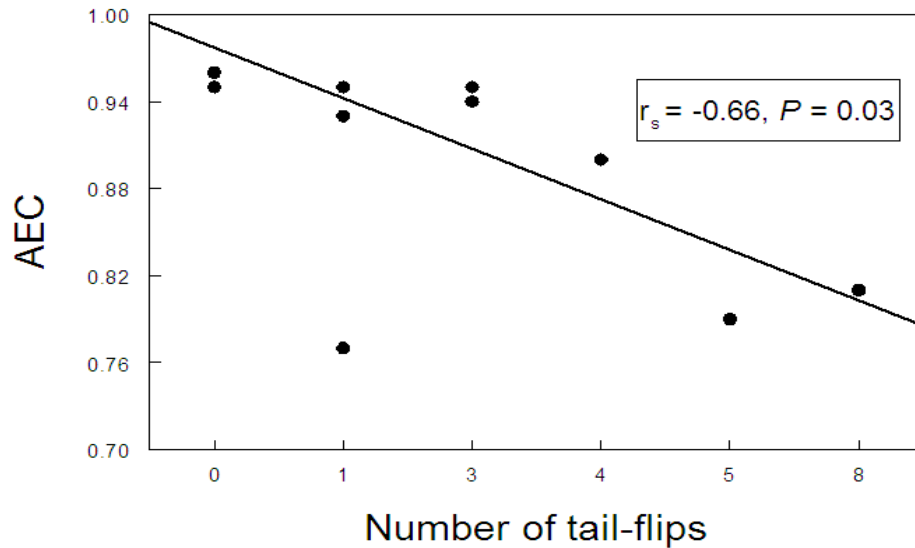


Fig. 1. Separation of nucleotides by HPLC. Mixture of standards (A) at a concentration of 1.8 μM IMP, 1.5 μM GDP, 1.8 μM AMP, 1.3 μM GTP, 1.7 μM ADP and 1.4 μM ATP. Sample of shrimp muscle (B) from group 3 (shrimp cooled in water at 5°C) of the experiment 2

that was frozen in liquid nitrogen after a short (<3 min) aerial exposure and crowding in the net. It is well known that hypoxia decreases AEC and ArgP in several crustaceans, although the effects were observed over longer time periods, ranging from minutes to hours (Gäde, 1984; Morris and Callaghan, 1998; Abe et al., 2007). Few studies have analyzed the particular influence of exposure to air; the effects

were observed over several hours, as is the case for *P. monodon*, for which AEC decreased from 0.9 to 0.67 when exposed to storage in sawdust at 12°C or 12 h (Paterson, 1993) or for Norway lobster (*Nephrops norvegicus*) with a decrease of AEC from 0.86 to 0.7 and a 65% decrease of ArgP when exposed to air during 2 h (Gornik et al., 2010). It seems likely that additional stress caused by tail flipping and crowding

A)



B)

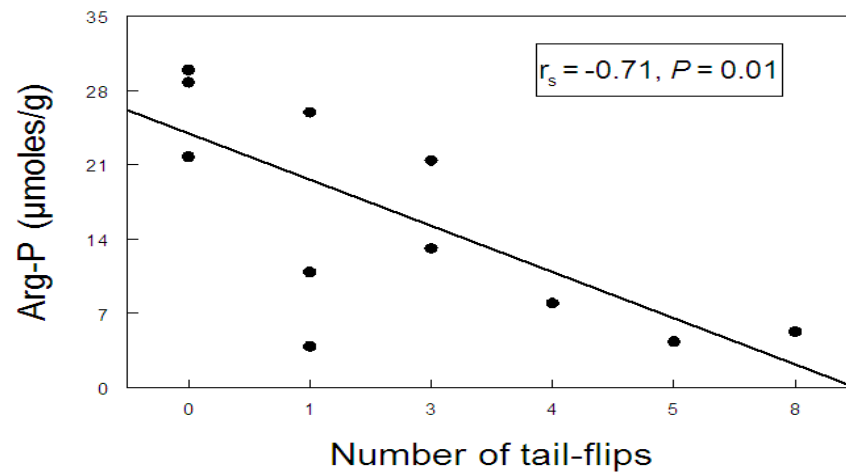


Fig. 2. Relation between AEC (A) or Arg-P (B) and the number of tail flips exhibited by shrimps during sampling.

were also important in our present conditions to obtain values of AEC as low as 0.61 and a 79% decrease of ArgP within a few minutes. This should be taken into account when capturing from ponds, estuaries and open sea with a net that will probably produce a similar effect.

Nevertheless, the main objective of the present experiment was to analyze cooling by other means than liquid nitrogen. In these cases, and although shrimp were massively captured, they were immediately placed in cold water or on ice; therefore, the results should be compared to individually frozen

Table 3. Effect of long-term storage at -76°C on the levels of different nucleotides and AEC in *Litopenaeus vannamei*

	Storage time				
	1 week	3 weeks	3 months	6 months	23 months
ATP	6.41 ± 0.51	7.14 ± 0.51	6.32 ± 0.55	7.45 ± 0.22	5.94 ± 0.72
ADP	1.24 ± 0.16	1.65 ± 0.19	1.60 ± 0.27	1.48 ± 0.35	1.59 ± 0.44
AMP	0.16 ± 0.05	0.32 ± 0.11	0.31 ± 0.11	0.23 ± 0.13	0.44 ± 0.27
TAN	7.81 ± 0.53	9.10 ± 0.29	8.23 ± 0.62	9.16 ± 0.35	7.97 ± 0.34
AEC	0.90 ± 0.02	0.87 ± 0.03	0.87 ± 0.02	0.90 ± 0.03	0.85 ± 0.06

See Table 1 for specifications.

Table 4. Levels of different nucleotides and AEC in *Litopenaeus vannamei* under different conditions

	Group 1 (recently transferred from ponds)	Group 2 (1 month in indoor tanks under suboptimal conditions)	Group 3 (Group 2 re-stocked under optimal conditions for 1 month)
ATP	9.43 ± 0.46 ^b	6.00 ± 0.62 ^a	7.19 ± 0.31 ^a
ADP	1.83 ± 0.35	3.03 ± 0.62	2.16 ± 0.21
AMP	0.32 ± 0.12	0.88 ± 0.39	0.42 ± 0.09
TAN	11.58 ± 0.51 ^b	9.91 ± 1.25 ^a	9.77 ± 0.30 ^a
AEC	0.90 ± 0.02 ^b	0.77 ± 0.04 ^a	0.85 ± 0.02 ^b
ArgP	16.74 ± 3.89 ^b	2.77 ± 0.34 ^a	9.08 ± 1.64 ^b

See Tables 1 and 2 for specifications

Table 5. Levels of different nucleotides and AEC in samples of muscle of *Litopenaeus vannamei* processed with different devices

	Bead beater	Rotor/stator	Ball mill mixer
ATP	7.05 ± 1.00 ^a (34.8) ¹	8.20 ± 0.48 ^{ab} (14.3)	9.78 ± 0.35 ^b (8.7)
ADP	2.56 ± 0.46 ^b (43.5)	2.11 ± 0.30 ^b (35.0)	0.98 ± 0.16 ^a (39.3)
AMP	1.22 ± 0.30 ^c (59.5)	0.39 ± 0.11 ^b (69.7)	0.07 ± 0.02 ^a (81.7)
TAN	10.83 ± 1.02 (23.2)	10.69 ± 0.44 (10.1)	10.83 ± 0.24 (5.4)
AEC	0.76 ± 0.04 ^a (12.0)	0.87 ± 0.02 ^b (6.7)	0.95 ± 0.01 ^c (2.6)

¹Coefficient of variation is indicated in parenthesis.

See Table 1 and 2 for other specifications.

See Methods section for additional details on devices used.

shrimp. In both groups, the AEC was lower, indicating that these procedures were not appropriate for sampling if liquid nitrogen is not available. The reduction of ATP by 50% and 73% in shrimps maintained in cool water and ice, respectively, was similar to fish, such as the gilthead seabream (*Sparus aurata*), in which ATP decreased by 50% in fish buried

in ice for 30 min compared to freshly dissected ones (Mendes et al., 2001).

It is well known that the decrease in ATP, together with a an increase in AMP or IMP (Table 2), are typical postmortem responses reported in several organisms even at 0°C, although these changes can oc-

cur within hours or days (Matsumoto and Yamakana, 1990; Mendes et al., 2001). The increase in IMP from deamination of AMP involves the key enzyme involved in nucleotide catabolism, AMP deaminase, which has much lower activity in invertebrates than in vertebrates (Lazou, 1989; Raffin and Thébault, 1991). Despite this low activity, the increase in IMP, in addition to AMP, occurs not only under postmortem conditions, but also in live crustaceans during stress situations, such as the escape response by tail flipping (Thébault and Raffin, 1991), air exposure (Paterson, 1993), and capture (Albalat et al., 2009), although in other cases it was undetectable under stress induced by tail flipping and exposure to air (Gornik et al., 2010). In our study, the increase in IMP was similar for both groups exposed 15 min to different cooling conditions (groups 3 and 4) and was not observed in shrimp that were exposed briefly (<3 min) to air (group 2). The pattern observed for IMP was therefore different than for AEC and ATP, for which similar levels were obtained between groups 2 and 3.

Changes observed in the different nucleotides seem to be caused by the kinds of stress (exposure to air, tail flipping, cold shock), rather than decomposition of shrimp, especially during such a short time. In shrimp ($n=3$) stored over two months at -20°C that were defrosted and refrozen once, we obtained values of AEC of 0.30 ± 0.03 and levels of ATP (0.04 ± 0.01), ADP (0.34 ± 0.04), AMP (0.37 ± 0.15), TAN (0.75 ± 0.20), and IMP (6.95 ± 1.17). These values, especially the low values of AEC, ATP and TAN, as well as the high values of IMP, reflected a decomposition process similar to those reported in other works (Matsumoto and Yamakana, 1990), and are markedly different from the values of groups cooled in water and ice.

In another trial, direct freezing of live organisms ($n=4$) in the -76°C ultrafreezer was also tested in a different experiment (data not shown) where an AEC value of 0.75 ± 0.11 was found. Therefore, the possibility to freeze shrimp directly into $-20/-30^{\circ}\text{C}$ in freezers that could be available near shrimp capture sites is also ruled out.

The strongest decrease in AEC and ATP, together with a more pronounced increase in AMP found in ice- compared to water-cooled shrimp, could be explained by the efficiency of cooling in relation to the conductivity of heat in air and water, together with the inherent stress of air exposure and escape response that occurred during the ice-cooling condition.

Postmortem depletion of ArgP over 3 h at 10°C was observed for the lobster *Nephrops norvegicus*, although a stronger decrease was observed after tail flipping (Gornik et al., 2010). In the present study, the similar depletion observed for the three groups also suggests that it could be attributed to stress rather than decomposition; additional stress involved in short-term storage on ice does not further deplete ArgP.

Long-term storage of shrimp at -76°C

Although the rapid freezing and storing of complete crustaceans is a common practice for subsequent nucleotide analysis, it is not specified for how long samples are stored; in general, samples are stored in liquid nitrogen at -196°C (Giesy et al., 1981; Moal et al., 1989) or at -80°C (Thomas et al., 2000). However, to our knowledge, no study of a possible time-storing effect on the adenylic concentration has been made. We observed in *L. vannamei* that long-term storage had no significant effect on individual or TAN concentration, or in AEC values that fluctuated between 0.85 and 0.90. Moreover, low values of AMP and undetectable IMP indicate that no decomposition of nucleotides was occurring.

Available information of nucleotide stability in stored crustaceans comes from studies at temperatures near 0°C that simulate storage for commercial purposes (Shimada et al. 2000, Albalat et al., 2011). In the particular case of penaeid shrimp, the levels of ATP in *M. japonicus* are almost depleted after 4 d of storage at -1°C (Matsumoto and Yamakana, 1990). It is known that the initial quality loss in aquatic organisms is primarily caused by postmortem autolytic changes, leading to the degradation of adenosine

nucleotides and other metabolites (Pacheco-Aguilar et al., 2008); however, enzymatic activity at temperatures below 0°C is reduced because of lower molecular interactions, protein denaturalization and increased viscosity. Others have pointed out that there is still some enzymatic activity below 0°C and that the molecular kinetics are not stopped until the temperature is below -65°C (Georlette et al., 2004; More et al., 1995; Sallam, 2007). It was reported that ATP decomposes at temperatures as low as -39°C (Jessen and Capplen, 1996, cited in Mendes et al., 2001). However, Thomas et al. (2000) mentions that storage temperatures below -40°C stop ATP catabolism indefinitely.

From these results, ATP degradation apparently does not occur in samples stored at -76°C for two years. This possibility offers the advantage to sample many organisms at a given time for further analyses later.

Baseline optimal condition of shrimp for sample collection

Shrimp transferred from ponds one day before presented significantly higher AEC and ArgP than shrimp stocked in clear indoor tanks at high density, with intermediate values for shrimp stocked in dark indoor tanks at lower densities. The differences in AEC were mainly caused by ATP levels in the three groups. The concentration of TAN in shrimp from ponds was significantly higher than in both groups in indoor tanks and higher than those obtained in the other experiments in outdoor tanks. Comparison of the three conditions was done because we observed low values of AEC under certain conditions of indoor tanks; therefore, we decided to compare shrimp under these conditions with shrimp from ponds and shrimp stocked in indoor tanks, but under less stressful conditions.

Lower values of ATP and TAN, but not of AEC, were also observed in the glass shrimp *P. paludosus* acclimated for three days to laboratory conditions compared to organisms from the field (Giesy et al., 1981), although no explanation was provided for

this result. In contrast, the transfer of the gastropod *P. ebeninus* from field to laboratory condition and stocked up to 6 weeks had no significant effect on AEC and TAN (Ivanovici, 1980b). Several possibilities can be suggested to explain the differences between pond and indoor tanks obtained in this study. Trophic levels may be one possible factor, as shown for oysters (Moal et al., 1991), and would result from natural food available in ponds, as well as higher availability of food in tanks at lower density. Chronic stress from captivity in indoor tanks, especially in small tanks at high density, can also explain lower AEC, pointing to its general significance as a chronic stress indicator (Ivanovici, 1980a). From several studies, it has been suggested that higher levels of ATP and ArgP were associated with higher energetic needs: i) seasonal variation in ATP content was related to increased motor activity occurring during the breeding period of the crayfish *Procambarus acutus* (Dickson and Giesy, 1982a); ii) a higher concentration of ATP in fasted surface crayfish *Procambarus clarkii* was probably linked to increased motor activity as a part of nutritional stress response, a response that was not observed in the cave crayfish *Orconectes inermis*, which have a lower metabolism (Dickson and Giesy, 1982b); iii) glass prawn *Palaemon elegans* has higher levels of ATP in concordance with a higher tolerance to environmental hypoxia compared to its close relative *Palaemon serratus* (Thébault et al., 1994). Therefore, it could be speculated that shrimp in ponds had higher levels of ATP and ArgP because they are more active with a higher metabolic rate compared to shrimp stocked in the laboratory under crowding conditions, although further studies should be done to test this hypothesis.

As mentioned in the Materials and Methods, shrimps from indoor tanks were first stocked in small tanks and then transferred to large tanks at a lower density. Whatever the particular reason for lower values of AEC and ArgP for shrimp kept in small tanks compared to recently transferred shrimps (stress, less food, or lower locomotor activity caused by crowding), we assume that a partial recovery of these variables occurs after removal of the stressor, in

accordance with previous studies (Ivanovici, 1980a; Marazza et al., 1996), or by providing more food.

Research on stress response in several decapod crustaceans, especially in shrimps and prawns, represents a methodological complication because there is inherent stress during sampling. For this reason, palaemonid prawns with spontaneous escape behavior were excluded from analysis (Thébault et al., 1994). The possibility that the spontaneous escape response (tail flipping) during shrimp capture could affect nucleotides and phosphagen levels was therefore examined. In the group of shrimp sampled one day after their transfer from ponds, i.e., the group with the highest values of AEC and ArgP, a significant correlation was obtained between AEC or ArgP against the number of tail flips (Spearman's correlation coefficient was -0.66 for AEC and -0.71 for ArgP), whether the shrimp were in water or nets (Fig. 2). This indicates that stress during sampling should also be avoided, although the effect is highly variable and further studies should analyze energy metabolism during tail flipping more thoroughly. In the present work, less than 30 s elapsed between the initial stimulus and immersion in liquid nitrogen. In accordance with these results, after approximately 90 s of induced tail flipping until exhaustion, reduced AEC and ArgP was observed under similar experimental conditions (unpublished results).

In shrimps from the experiment with long-term storage, it was also possible to test if stress induced by sampling on the remaining shrimp in tanks affects the AEC. Indeed, an increase in blood glucose and lactate concentration occurred in fish under these conditions (Molinero et al., 1997; Racotta et al., 2004).

Tissue processing: different methods of homogenization

In addition to an adequate sampling and preservation of the samples, the initial sample processing is a critical step that could affect levels of nucleotides. Among the three devices tested, each has advantages and disadvantages. The bead beater (model FastPrep24, MP

Biomedicals) processes 24 samples at the same time. For the bead beater and the rotor/stator homogenizer (model Tempest IQ2, the VirTis Company), liquid nitrogen is not necessary. In contrast, operation of the ball mill mixer (model MM400, Retsch) requires liquid nitrogen; its advantage is that a homogeneous powder is obtained without a problem of differential anatomical sampling in terms of kinds of muscle fibers. In addition, the powder can be used for other analyses without reprocessing another muscle sample. However, the best procedure should be based on the lowest loss of nucleotides (specific or total) that are tested in the experiment.

Lower values of AEC were obtained by using the bead beater compared to the ball mill mixer, with intermediate values for the rotor/stator homogenizer (Table 5). The lower AEC was mainly due to the decrease of ATP, increase of AMP, and to a lesser extent, to the decrease of ADP. These results clearly indicate that the bead beater should be discarded for the analysis of nucleotides, mainly because the samples were not maintained in cold conditions, unlike the other two devices. Test tubes used for homogenizing the samples with the rotor/stator device are maintained on ice, which is not possible with the bead beater because all tubes are introduced into the apparatus that functions at room temperature. The homogenization is performed under acidic conditions, and therefore the acidification of the sample/precipitation of proteins inactivates the enzymatic processes that reduce ATP levels and AEC. However, this seems to occur less efficiently with the bead beater because cooling is absent. Possibly, there is a slower disaggregation of tissue and penetration of acid into tissue from the mechanical process. Among the few existing studies on the influence of sample processing, Moal et al. (1989) demonstrates the advantage to prior grinding of oysters with a ball mill mixer to obtain tissue powder compared to direct homogenization of frozen oysters with a Potter device, which resulted in lower values. On the other hand, Thomas et al. (2000) clearly showed the lability of ATP during processing by obtaining much lower values of ATP in fish samples macerated with a scalpel on a Petri glass on ice than samples processed under cryogenic condition.

Significant differences were obtained between the rotor/stator homogenizer and ball mill mixer for AEC, ADP and AMP. In contrast, for oyster tissues, no differences were obtained between similar devices, from different suppliers (Racotta and Moal, unpublished results). Nevertheless, the difference of AEC values between the devices used in the present work is quantitatively minor and within the overall variation obtained for the different experiments. In our study, the rotor/stator homogenizer was used for the first three experiments (experiments on sampling and experiment on extended storage) and the ball mill mixer was used in the fourth experiment on shrimp condition. With both devices, values of AEC above 0.9 were obtained and therefore we assumed that no degradation of ATP occurred during sample processing. On the other hand, shrimp used in the comparison of devices were those transferred from ponds one day before testing, i.e. they had AEC and ATP values of 0.9 and 9.4, respectively when processed with a ball mill mixer (see Table 4), which are intermediate values between both devices (Table 5). This suggests that the difference between the two devices partially results from biological variation. The difference attributed to sample processing itself is also probably related to sample heating, which would also explain high variability with such devices. Hence, we concluded that, depending on availability, both could be used. Another important criterion may be the coefficient of variation obtained with the devices that in general is lower for the ball mill mixer and therefore indicates that this device is more accurate.

CONCLUSIONS

We conclude that the best sampling procedure to obtain baseline values for shrimp is either immediate, freshly dissected and homogenized tissue in acid or immersed in liquid nitrogen and stored at -76°C for up to two years, provided that the freezing procedure is rapid enough to avoid ATP hydrolysis. Other cooling procedures that are more common on shrimp farms, such as immersion in cold water or placing shrimps on ice for 15 min or even introducing shrimps directly to a freezer, are not adequate

alternatives. When transferred to indoor tanks, optimal conditions of stocking densities, tank color and tank dimensions should be provided to obtain an energy status inferred from AEC and ArgP levels representative of healthy and non-stressed shrimp. These results suggest that such variables constitute a good index for acute and chronic stress conditions and should be tested under practical conditions in which an altered energy status predicts an adverse condition associated with a lower performance.

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