Response of the blue shrimp Litopenaeus stylirostris to temperature decrease and hypoxia in relation to molt stage

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Abstract:

The effect of different levels of dissolved oxygen (DO) and a decreased temperature combined with severe hypoxia were studied in the blue shrimp *Litopenaeus stylirostris* in relation to molt stage. In the first experiment, shrimps were submitted to DO concentrations of 5 to 1 mg l^{-1}. Osmoregulatory capacity (OC) was measured in shrimps at molt stage C after 6, 24, or 48 h of exposure. No mortality was recorded but a significant negative effect of DO concentration on hypo-OC was observed for DO concentrations below 3 mg l^{-1}. Osmoregulation improved after 48 h exposure, compared to 6 and 24 h exposure.

In a second experiment, temperature was decreased from 28 to 22 °C in 24 h, before a severe hypoxia of 0.5 ± 0.1 mg O_2 l^{-1} was applied. Mortality and OC were studied in relation to molt stages. Half of the shrimps died after respectively 100 min at 0.5 mg O_2 l^{-1} at 28 °C and 153 min at 0.4 mg O_2 l^{-1} at 22°C. Mortality was significantly higher in stages D_2 and B compared to stages C and D_0. Control shrimps in stage D_2 had a lower hypo-OC than shrimps in stages C and D_0 both at 28 and 22 °C. Temperature decrease had no effect on hypo-OC. Hypoxia reduced hypo-OC for all stages, whatever the temperature, but the effect was greater in stage C at 27 °C (68% decrease) compared to 22 °C (49%) and stage D_2 at both temperatures (respectively, 43% and 58% at 27 and 22 °C). Combination of temperature and hypoxia had a significant effect on hypo-OC. Low temperature reduced the effect of hypoxia presumably by slowing down the metabolism. Results are discussed in relation to pond observation.

1. Introduction

Shrimp farming in New Caledonia is dedicated to the blue shrimp <u>Litopenaeus stylirostris</u> in earthen ponds. Although it is a developing industry, it is facing episodes of mortality due to a pathogenic bacteria <u>Vibrio penaeicida</u>. The disease appears mainly at the inter-season periods, although most of the shrimps carry the <u>Vibrio</u> throughout the years and are apparently healthy (Goarant, pers. comm.). Drops of water temperature seem to have an impact on mortalities (Mermoud et al., 1998) but are not sufficient to trigger off the disease. Other external and/or internal (such as molt stage) factors may be implied. Dissolved oxygen (DO) concentration in ponds is one of the main environmental factors which often undergoes large fluctuations and can induce a stress in shrimp, especially in semi-intensive culture system for which no air supply is provided in the ponds. During the last phase of grow-out in ponds without aeration, shrimps can be punctually exposed to short-term hypoxia (oxygen level from 3 mg l^{-1} to less than 1 mg l^{-1}). Short-term decreases of DO concentration can have a negative effect on shrimp immune system (Direkbusarakom and Danayadol, 1998; Le Moullac et al., 1998; Le Moullac and Haffner, 2000), and may decrease resistance to diseases. It has also been shown that <u>L. stylirostris</u> subjected to diurnal cycles of 1.5 mg O_2 l^{-1} to saturation presented lowered survival and growth, and their molting cycle was modified (Aquacop et al., 1988). Long-term hypoxia can affect molting (Clark, 1986) and growth (Ocampo et al., 2000). It has also been shown that depending of their molt stage shrimps can be more or less sensitive to stress (Wajsbrot et al., 1990), or experimental infection with pathogenic bacteria (Le Moullac et al., 1997; Cheng et al., 2003).

In the present study, two experiments were conducted in order to evaluate the effect of (1) DO concentrations and (2) a temperature decrease followed by hypoxia, on the survival and physiological status of juvenile <u>L. stylirostris</u> in relation to molt stage. In a first experiment juvenile <u>L. stylirostris</u> were subjected to different DO concentrations and sampled after 6, 24 or 48 hours of exposure, in order to define from which DO concentration, in relation to duration of exposure, shrimps become physiologically affected. In a second experiment, <u>L. stylirostris</u> were subjected to severe hypoxia at two different temperatures. The decreasing temperature applied in the experiment corresponded to natural variations observed in ponds during inter-season in New Caledonia. In both experiments mortality was recorded and osmoregulatory capacity (OC), which is a sensitive indicator for detecting physiological stress

(Lignot et al., 2000), was measured in intermolt (experiment 1 and 2) and late premolt shrimps (experiment 2). OC is the difference in osmotic pressures between haemolymph and surrounding water at a given salinity (Charmantier et al., 1989). Osmoregulation, which maintains osmotic homeostasis, is one of the main regulatory functions in aquatic animal (Péqueux, 1995). L stylirostris is a good hypo-osmoregulator in sea water which OC may be affected by stressors such as ammonia exposure (Mugnier and Justou, 2004), organophosphorus insecticide exposure (Lignot et al., 1998) or low temperatures (Lemaire et al., 2002).

2. Material and methods

2.1. Molt stage determination

Six molt stages were defined according to the retraction of the epithelium within setae of the antennal scale (Drach, 1939; Chan et al., 1988). Shrimps were classified as A and B for respectively the early and late postmolt stages, C for intermolt and D_0, D_1, D_2 for premolt stages. D_2 was the late premolt stage prior to ecdysis, when epidermis is at maximal retraction and it is possible to distinguish the developing seta.

2.2. Experimental animals

The experiments took place in the Tropical Aquaculture Laboratory of IFREMER in Tahiti (French Polynesia). L. stylirostris were reared in earthen pond. Juvenile shrimps of 9-10 g weight were transferred to indoor tanks containing 75 l of sea water, which was recirculated (experiment 1) or either recirculated or flow-through (8 tanks each, experiment 2). Densities were respectively 45 and 22 shrimps/tank in experiments 1 and 2. Temperature was 28°C, salinity 36‰ and the osmotic pressure of the water 1057 mOsm kg^{-1} in both experiments. Shrimps were fed commercial pellets. They were not fed 12 hours before, and during, the experiment. Juvenile shrimps were acclimated for at least 2 days before experiments started (Soyez, 1997).

2.3. Effect of different dissolved oxygen concentrations on mortality and osmoregulatory capacity

DO concentration was maintained to saturation (6-7 mg l^{-1}) before the experiment started and during the experiment for control shrimps. For challenged shrimps, the desired DO concentrations were the result of shrimp oxygen consumption. The surface of the water was covered with a plastic film to limit gas exchange at the surface. Required DO concentration was maintained by continuous monitoring of oxygen pressure (PO_2) with an Orbisphere oxymeter by opening or closing air electrovalves to adjust oxygen level. DO concentrations were maintained at 5, 4, 3, 2 and 1 mg O_2 l^{-1}. Shrimps were maintained during 6, 24 or 48 hours at the concentrations cited. Mortality was recorded at the end of the experiment. Haemolymph samples of shrimps in stage C were collected via the ventral sinus using disposable syringes and needles. Osmotic pressure of haemolymph was immediately measured on a Wescor 5500R vapor pressure osmometer. Osmotic pressure of the water was also measured for the calculation of OC.

2.4. Effect of temperature decrease and severe hypoxia on mortality and hypo-OC

The experimental procedure is shown on fig. 1. Temperature was gradually decreased from 28°C to 22°C in 24h in 8 tanks out of 16. Dissolved oxygen level was maintained between 6 and 7 mg.l^{-1} by constant aeration before the experiment started for all tanks, and during the experiment for control tanks at both 28 and 22°C. The decrease of DO concentration (4 tanks for each temperature) was the result of shrimp oxygen consumption after the supplies in water and air were stopped, and the surface of the water covered with a plastic film to limit gas exchange at the surface. Shrimps were sampled for osmotic pressure as in experiment 1 in two tanks out of 4 for each treatment when DO concentration reached 0.6 mg l^{-1}. Because there were not enough animals at stages A, B and D_1 either at 22°C or at 28°C, only shrimps in stages C, D_0 and D_2 were sampled. Oxygen concentration was frequently monitored using an Orbisphere oxymeter in the remaining tanks. Each time a dead shrimp was observed in the tanks it was removed and the molt stage determined. The experiment was stopped when half of the shrimps were dead. The molt stage of the survival shrimps was also recorded. Control shrimps were sampled at the end of the experiment.
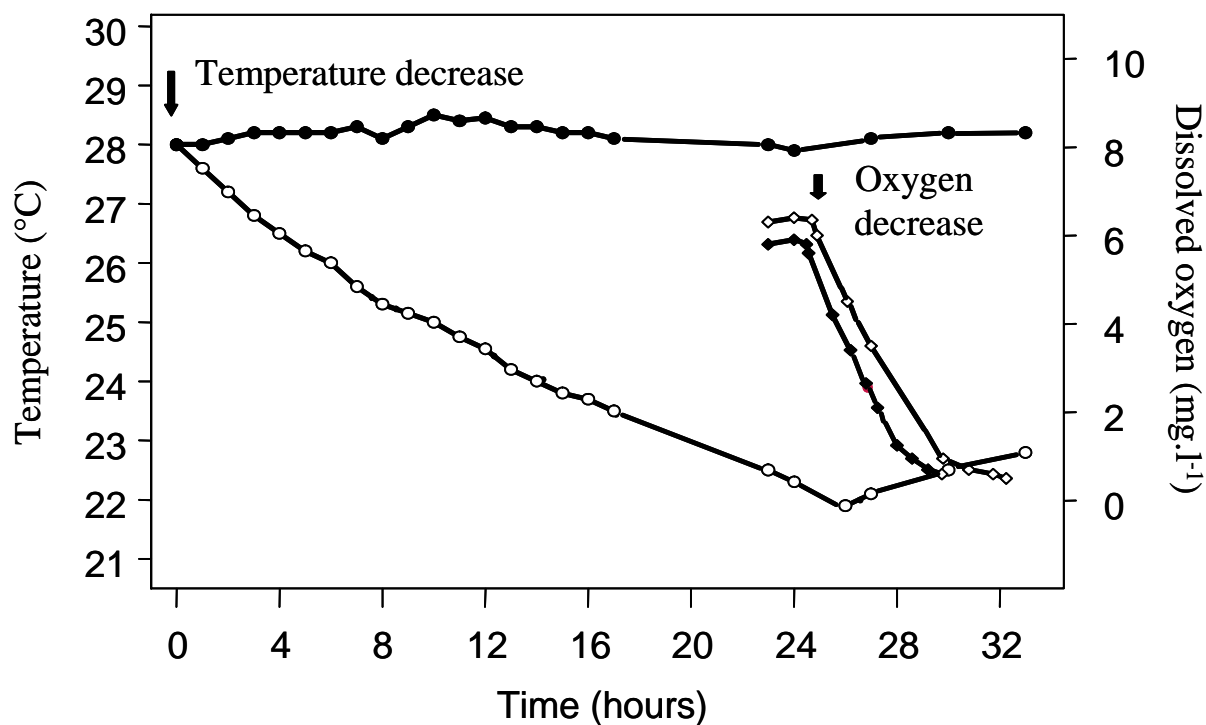
Fig. 1. Kinetic of experimental temperature and oxygen decreases applied on L. stylirostris in the second experiment. Open symbol: 22°C; black symbols: 28°C.

2.5. Data and statistical analysis

The OC was calculated as the difference between the osmotic pressure of seawater and shrimp haemolymph. The data were expressed as the mean ± standard error of the mean, except for mortality expressed in percentage. Data of the first experiment are shown as the depression of OC compared to the control for each time studied. Data were analysed with two-way and one-way ANOVA followed by the PLSD Fisher test at the significant threshold of 5% (Statview computer software). Differences in mortality between shrimp at different molt stages were analysed by Chi-square tests at the level of significant threshold of 5%.

3. Results

3.1. Effect of different dissolved oxygen concentrations (figure 2)

No mortality was recorded during the experiment. There was a significant effect of DO concentration on hypo-OC (p<0.0001). A decrease in hypo-OC of shrimps maintained at 1 and 2 mg O_2 l^{-1} was observed compared to shrimps maintained at 3, 4, 5 and 6 (control) mg O_2 l^{-1}. Duration of exposure to the different DO concentrations had also a significant effect on hypo-OC (p<0.0001) for DO concentrations below 3 mg l^{-1}. Hypo-OC was significantly higher after 48 hours exposure than after 6 and 24 hours. The combined effect of DO level with duration of exposure was significant (p=0.0035).
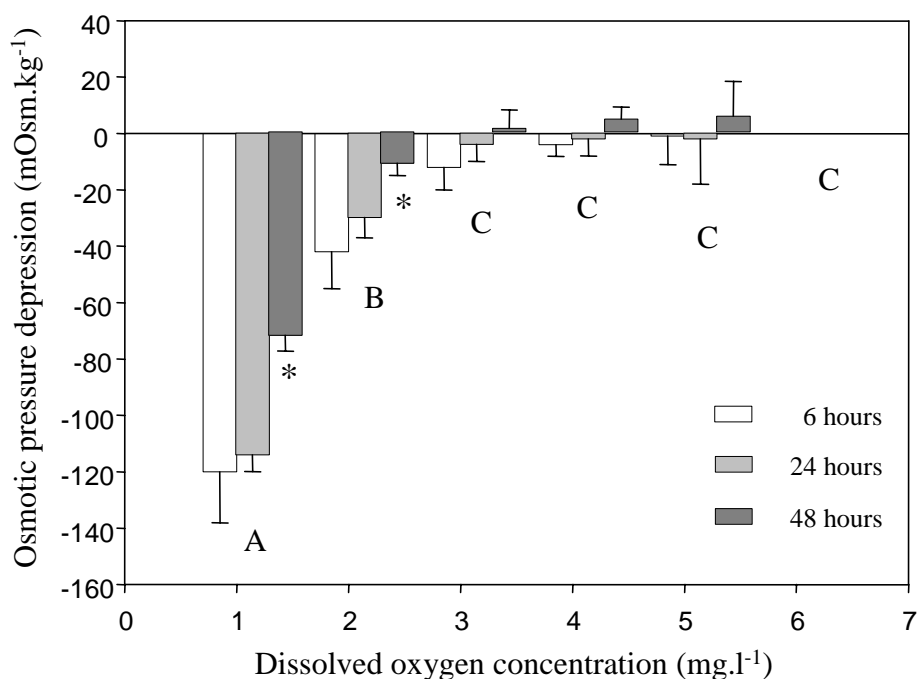

Fig. 2. Osmotic pressure depression (mean ± S.E.) in L. stylirostris submitted to different dissolved oxygen level for different times of exposure in comparison with the control groups maintained at 6 mg O_2 l^{-1}. Significant differences between groups are indicated by different capital letters (P<0.05). *Bars within group are significantly different (P<0.05).

3.2. Effect of hypoxia and temperature decrease

No mortality was recorded in tanks maintained under normoxia at both 28°C and 22°C. A reduction in locomotry activities was observed as DO concentration declined in hypoxic conditions. Below 1.5 O_2 mg l^{-1}, all the shrimps got together and stayed very still. Mortality started 24 min after oxygen level reached 0.6 mg l^{-1} at 28°C and after 135 min at 22°C when oxygen level reached 0.45 mg l^{-1}. It took 100 min at 28°C and 153 min at 22°C to have 50% of the shrimps dead. DO concentration in tanks maintained at 22°C was as low as 0.35 mg l^{-1} when the experiment was stopped. The initial temperature of 22°C increased slightly to 22.5°C when DO level was close to 0.6 mg l^{-1}, and 22.7°C at the end of the experiment.

Mortalities in relation to molt stages are presented on figure 3. No animals in stage D_2 were observed at 22°C. There was no significant differences (p=0.920) between the other stages. Mortality was significantly higher for shrimps in advanced premolt stage D_2 and postmolt stage B than for intermolt and early premolt shrimps (C and D_0) at 28°C.
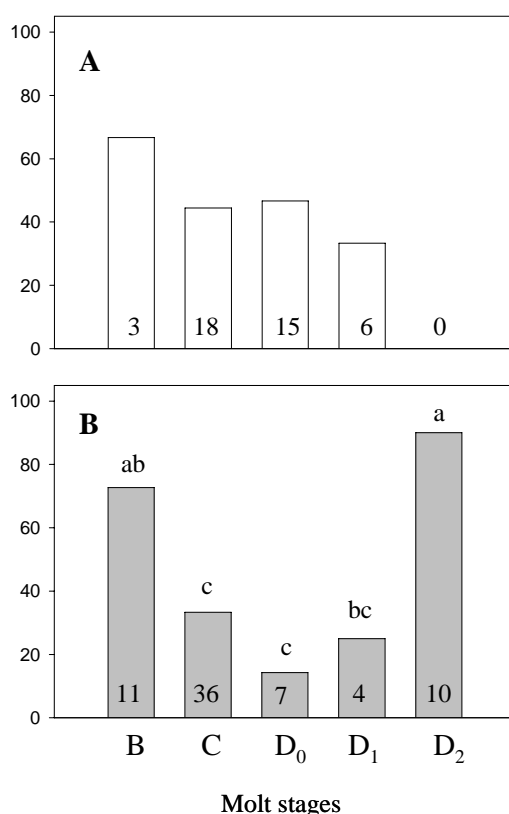

Fig. 3. Percentage of mortality for each molt stage at 22°C (A) and 27°C (B) in L. stylirostris submitted to hypoxia. Number of animals are indicated into bars. Bars with different letters are significantly different (p<0.05).

Hypo-OC values of shrimps in relation to treatment and molt stage are shown in Table 1. Temperature decrease had no significant effect on hypo-OC in shrimps maintained under normoxia whatever the molt stage. Mean hypo-OC of shrimps in stage D_2 under normoxia was significantly lower than that of shrimps in stages C and D_0 at both temperatures. The difference was 14% at 28°C and 11% at 22°C. No significant differences were observed between molt stages in shrimps under hypoxia, whatever the temperature. Hypoxia led to a significant decrease of hypo-OC for all stages, whatever the temperature. Stage (p=0.0073), treatment (p<0.001) and combination of both (p=0.006) had a significant effect on hypo-OC. Hypo-OC of shrimps in stage C under hypoxia was significantly lower at 28°C compared to 22°C (Table 1). No difference was observed for shrimps in stage D_2. Comparison was not possible for stage D_0 (no data). For animals in stage C, hypoxia led to a 68% decrease of hypo-OC at 28°C compared to the control, and 49% at 22°C. Decreases were respectively 43% and 58% for shrimps in stage D_2, and 53% for stage D_0 at 22°C.

Table 1

Hypo-osmoregulatory capacity ($mOsm.kg^{-1}$) in relation to temperature and oxygen treatment of <u>L. stylirostris</u> according to the molt stage (experimentation 2) (mean ± S.E, (n))

	28°C		22°C		
	Normoxia	Hypoxia	Normoxia	Hypoxia	
Stage C	-232.7±13.5 [a]	-73.9±10.7 [c]	-228.6±7.5 [a]	-116.4±8.8 [b]	P<0.0001
	(3)	(18)	(10)	(10)	
Stage D0	-214.2±6.9 [a]	-	-220.6±6.8 [a]	-116.4±15.6 [b]	P<0.0001
	(11)	(0)	(13)	(5)	
Stage D2	-181.5±9.2 [a*]	-102.6±11.2 [b]	-199.0±6.1 [a*]	-83.4±7.8 [b]	P<0.0001
	(15)	(8)	(16)	(7)	
	*P<0.05	P>0.05	*P<0.05	P>0.05	

Significant differences for each molt stage are shown by different letters

* indicates difference between molt stages for each treatment

4. Discussion

4.1. Effect of oxygen depression

In experimental conditions, DO concentrations from 3 mg O_2 l^{-1} and above had no effect on osmoregulation in juvenile L. stylirostris (stage C). A significant negative effect of DO concentration on hypo-OC was observed for concentrations below 3 mg O_2 l^{-1}, as it was also observed in P. vannamei (Charmantier et al., 1994). However, no mortality was recorded over the 48 hours, while Allan and Maguire (1991) observed 16.7% mortality over 48 h of exposure to 1 mg O_2 l^{-1} in P. monodon. In the present study, shrimps presented a maximum stress response (lowest hypo-OC) after 6 hours of exposure to low DO concentration, and this response seemed to be maintained after 24 hours exposure. However this negative effect tended to decrease with time, as shown by a higher hypo-OC after 48 hours exposure, possibly because the shrimps adapted partially to the low concentration of DO. Moreover, Soyez (1997) showed that L. stylirostris exposed to a constant DO of 2 mg l^{-1} over a month did not show any mortality nor reduction in growth, while P. semisulcatus exposed to a long-term DO stress of 2 mg O_2 l^{-1} for 17 days, showed mortality and molting was inhibited (Clark, 1986). These results seem to indicate that L. stylirostris is relatively well resistant to hypoxia. However, subjected to diurnal-like cycles of 1.5 mg O_2 l^{-1} to saturation, L. stylirostris presented lowered survival and growth, and their molting cycle was modified (Aquacop et al., 1988). The results of the present study coupled to those of Soyez (1997) and Aquacop et al. (1988) tend to indicate that repetition of stress such as a low DO level is more stressful than a single acute or a long term hypoxia. The results of Allan and Maguire (1991), who did not observe any reduction in growth over 21 days in P. monodon exposed to a single acute DO-stress enhance the limited effect of an acute stress.

No mortality was recorded for a DO concentration of 1 mg l^{-1} at 28°C, even after 48h of exposure. However, as shown in experiment 2, less than 30 min at 0.6 mg O_2 l^{-1} seems to be the tolerance limit before mortality started at this temperature. In less than two hours half of the shrimps were dead. It is quicker than the LT_{50} observed for P. monodon (16h at 0.5 mg O_2 l^{-1}, 27.1 °C) (Allan and Maguire, 1991), or Metapenaeus ensis (399 min at 0.5 mg O_2 l^{-1}, 22°C) (Wu et al., 2002), but experimental conditions are different and make the comparison difficult as in the present study the shrimps were under stressful DO level (less than 3 mg O_2 l^{-1}) 6 hours before the level reached 0.6 mg O_2 l^{-1}. In shrimp pond such a continuous decrease

of DO level is observed during the night, due to the consumption of oxygen by phytoplankton. The present results indicate that consequences could be dramatic if oxygen level decrease below 1 mg O_2 l^{-1} and does not go up to 3 mg O_2 l^{-1} quickly enough, all the more since it is repeated for several days or happening when most of the shrimp are molting.

4.1. Effect of temperature decrease

In our experimental conditions, a temperature decrease of 6°C in 24 hours had no effect neither on survival nor ionic regulation in both pre- and intermolt shrimps, while in pond such a decrease till 22°C is often followed by mortality outbreaks. The main hypotheses concerning the origin of the difference of response between pond and experimental facilities are: (1) the repetition of such temperature decreases due to diurnal variations in pond could be more stressful than a single isolated decrease as observed for DO (Aquacop et al., 1988), and/or (2) the temperature effect could be toward the pathogen (increase in number and/or virulence) (Goarant et al., 2000), and/or (3) associated with the pathogen: an environmental stress following an experimental infection with a pathogenic bacteria may increase mortality (unpublished data) and/or eventually (4) the temperature decrease could be associated with other stressful factors and act in synergy. Indeed, levels of one or more parameters such as DO, ammonia, pH, or nitrites in ponds may be close to the tolerance limits of the shrimp as the same time as the temperature decrease (Boyd, 1982) and may have a synergic effect. For instance, it was observed that DO levels below 55% saturation increased toxicity of ammonia to juvenile P. semisculatus (Wajsbrot et al., 1990). A comparable result was obtained by Allan et al. (1990) on P. monodon.

4.2. Combined effect of temperature decrease and hypoxia

Association of oxygen depression with a lower temperature had no effect (stage D_2) or a positive effect (stage C) on shrimp osmoregulation and tolerance to hypoxia compared to 28°C. A lower temperature seems to increase tolerance of shrimps to hypoxia, as shown by a hypo-OC which was less depressed at 22°C than at 28°C. This effect was not observed for stage D_2 for which the hypo-OC was already lower at 28°C compared to stage C. It was previously observed for L. stylirostris (Lignot et al., 1999; Mugnier and Justou, 2004) and for the shrimp P. vannamei (Charmantier et al., 1994) that premolt shrimps (stage D_2) have a lower hypo-OC than intermolt animals at DO saturation level. The increase in osmolality could be associated to the beginning of a water uptake at molt. The lack of negative

combined effect of temperature decrease and hypoxia could be due to a slower metabolism and a decrease in oxygen need at 22°C, as it was observed in the freshwater prawn Macrobrachium rosenbergii. In this species, a 30% decrease of oxygen uptake was observed between 27°C and 22°C (Chen and Kou, 1996). It is observed in general that power of metabolic regulation tends to decrease with increase of ambient temperature, possibly in relation to an increase of metabolism and limits due to the adaptability of oxygen-carrying systems from ambient medium to the cells (Truchot and Jouve-Duhamel, 1983). However, there are species like the shrimp P. japonicus for which on the contrary regulation power increases till temperatures around 26-30°C (Truchot and Jouve-Duhamel, 1983), but L. stylirostris does not seem to follow this model. In hypoxic conditions, premolt shrimps do not seem to be more sensitive to oxygen stress than intermolt shrimps, in term of hypo-OC depression, possibly because the osmoregulation is already lower for this stage compared to intermolt animals. However it has been shown in M. rosenbergii that oxygen consumption increased during the premolt and molt stages (Stern and Cohen, 1982). The authors suggested that more carbone is being utilized for metabolic requisites, and not for tissue growth and organic reserve accumulation as in the intermolt stage. However, a higher mortality rate in our experiment indicates that D_2 shrimp are more affected by hypoxia than intermolt animals at 28°C.

DO levels below 3 mg O_2 l^{-1} are observed in ponds during the inter season when temperature drops and mortalities occur. The present results tend to show that the combination of both factors can not explain the mortality outbreaks. It confirms the probable pluri-factorial and complex origin of these outbreaks and that more studies are needed, particularly on the relation between the pathogen, the shrimp and the environment.

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