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ANISSA BEN RADHIA - SOUISSI

Etude de la plasticité reproductive et de la morphologie d'un copépode estuarien: comparaison intercontinentale

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Thèse dirigée par SAMI SOUISSI & JIANG-SHIOU HWANG

Soutenue le 14 décembre 2010 à Wimereux devant le jury composé de :

Michèle TACKX, PR Université de Toulouse	Rapporteur
Joëlle FORGET-LERAY, PR Université du Havre	Rapporteur
Isabella BUTTINO, DR Station Zoologique de Naples	Examinateur
Xavier VECKEMANS, PR Université de Lille 1	Examinateur
Jiang-Shiou HWANG, PR National Taiwan Ocean University	Directeur de thèse
Sami SOUISSI, PR Université de Lille 1	Directeur de thèse

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Résumé

Dans le cadre de cette thèse nous avons mis au point un nouveau protocole expérimental multi-générationnel permettant d'étudier d'une façon très précise la plasticité du copépode *Eurytemora affinis* dans le cadre d'un scénario de changement climatique. Nous avons montré que cette espèce cryptique, à large répartition géographique dans l'hémisphère nord, est capable de se développer à des fortes températures et/ou salinités. Nous avons souligné l'importance du contrôle maternel mais également les effets trans-générationnels sur les traits de cycle de vie d'*E. affinis*. Dans des conditions expérimentales contrôlées, trois populations d'*E. affinis* d'origines différentes ont montré des réponses similaires en termes de survie et de fécondité. Toutefois, nous avons confirmé que la population d'*E. affinis* provenant de l'estuaire de la Seine se caractérise par un sex-ratio déséquilibré en faveur des males. Cette observation qui s'ajoute à toutes les précédentes études *in situ* semble confirmer que le déterminisme sexuel chez cette population pourrait avoir une origine génétique. En effet, lors d'un épisode de chute de la qualité nutritive des algues, seule la population d'*E. affinis* de la Seine a montré, à faible température, des individus intersexués (mâles féminisés). Compte tenu de l'importance de la reproduction dans la dynamique de population des copépodes, nous avons étudié d'une façon détaillée la stratégie reproductive des femelles, des mâles ainsi que de leur interaction. Nous avons mis en évidence, pour la première fois en laboratoire mais également *in situ*, la présence de «trade-off» (compromis) entre la fécondité (nombre d'œufs) et la taille de l'œuf chez une espèce de copépode. Les femelles d'*E. affinis* sont capables d'optimiser leur allocation d'énergie reproductive en augmentant leur investissement dans la qualité de l'œuf (et donc de leur descendance) à faible température. Nous avons également montré une nette différence entre les populations transatlantiques d'*E. affinis* avec les populations européennes où la femelle est relativement plus longue et produit des œufs plus volumineux alors que les femelles des populations Nord-américaines sont plus larges et produisent des œufs de plus petites tailles. Nos nouveaux résultats, combinés à d'autres observations morphologiques fines, semblent confirmer l'aboutissement du processus de spéciation au sein de cette espèce cryptique. En plus des différences morphologiques et génétiques, nous avons démontré pour la première fois, les conséquences de la spéciation au niveau de la stratégie reproductive qui oppose les populations transatlantiques d'*E. affinis*.

Enfin, l'analyse détaillée du rôle du mâle, souvent négligé, et du rôle de la femelle dans la reproduction des copépodes a été illustrée pour la population d'*E. affinis* provenant de l'estuaire de la Seine. Nos résultats ont révélé que ; le mâle contribue activement à la reproduction puisqu'il est capable d'identifier les femelles prêtes à s'accoupler (avec des ovocytes matures), la femelle est capable de s'accoupler avec plusieurs mâles et d'utiliser pour une même portée le matériel génétique de plusieurs pères. Il semble qu'un sex-ratio en faveur des males offre des avantages avérés à la population d'*E. affinis* de l'estuaire de la Seine.

En conclusions, plusieurs résultats obtenus dans le cadre de cette thèse dépassent le cadre des études en écologie marine et confirment le grand potentiel de ce copépode afin de le promouvoir en tant que modèle biologique à l'image des modèles connus en écologie terrestre. Nous avons souligné l'importance d'intégrer la plasticité des organismes vivants et leur capacité d'adaptation aux variations de la température ainsi que d'autres facteurs environnementaux dans le cadre du débat actuel sur les effets du changement climatiques sur les écosystèmes.

Mots clés: *Eurytemora affinis*, multi-génération, changement global, plasticité, taille, stratégie reproductive, fitness, trade-off, comportement reproducteur.

Abstract

In the framework of this thesis, we finalized a new multigenerational experimental protocol allowing the study, in a very precise way, the plasticity of the copepod *Eurytemora affinis* within a climate change scenario. We showed that this cryptic species, in wide geographical distribution in the north hemisphere, was capable of developing at strong temperatures and\or salinity. We underlined the importance of the maternal control but also the trans-generational effects on the life cycle traits of *E. affinis*. Under controlled experimental conditions, three populations of *E. affinis* from different origins showed similar answers in terms of survival and fertility. However, we confirmed that the Seine estuary population of *E. affinis* is characterized by male biased sex-ratio. This observation, in addition to all the previous *in situ* studies seems to confirm that the sexual determinism of this population could have a genetic origin. Indeed, during an episode of low algal food supply quality, only the population of *E. affinis* from the Seine showed intersexual individuals (feminized males), at low temperature. By considering the importance of the reproduction in the dynamics of the copepod population, we studied, in a detailed approach, the reproductive strategy of females and males as well as their interaction. We inderlined, for the first time in laboratory but also *in situ*, the presence of a trade-off between the fertility (number of eggs) and the egg size of a copepod species. *E affinis* females were capable of optimizing their energy reproductive allocation by increasing their investment in the quality of the egg at low temperature. We also showed a clear difference between the transatlantic populations of *E. affinis* with relatively longer females producing more voluminous eggs in the European populations and wider females producing smaller size eggs in the North American populations. Our results, combined to other fine morphological observations, seem to confirm the outcome of the speciation process within this cryptic species. Besides the morphological and genetic differences, we demonstrated for the first time, the consequences of the speciation at the reproductive strategy level within the transatlantic populations of *E. affinis*. Finally, the detailed analysis of the male's role that was often neglected, and the role of the female in the copepod reproduction was illustrated for the *E. affinis* population from the Seine estuary. Our results revealed that; the male contributes actively to the reproduction since he is capable of identifying females ready to mate (with mature oocytes), the female is capable to mate with several males and to use the genetic material of several fathers for the same clutch. It seems that a male biased sex-ratio offer advantages to the E. affinis population of the Seine estuary. In conclusions, several results obtained in this thesis confirmed the high potential of this copepod species to be promoted as a biological model like those well established in terrestrial ecology. We underlined the importance of considering the plasticity of living organisms and their adaptation capacity to temperature variations as well as other environmental factors in the current debate of the impact of global changes on ecosystems.

Keywords: *Eurytemora affinis*, multi-generation, global change, plasticity, size, reproductive strategy, fitness, trade-off, reproductive behavior.

General Introduction

Zooplankton and especially copepods are probably the most abundant animals on earth (Humes 1994; Boxshall and Halsey 2004) playing a key role in most aquatic ecosystems of our planet. Although their importance, copepods were often studied empirically and the use of the descriptive approaches almost dominated the literature. The recent studies that tried to propose general relationships in a more global framework showed that temperature, food and the size of copepods are the main variables shaping the life cycle traits (Hirst and Bunker, 2003; Bunker and Hirst, 2004). However, these studies, based on the compilation of data from the literature suffred from a high dispersion and luck of unified ecological and evolutionary framework. For example, under the current debate of the possible effects of climate change on ecosystems, it is difficult to predict the responses of copepods to any given scenario (i.e. global warming). In fact, these short living organisms are characterized by a high plasticity and would certainly show a complex rather than a direct response to temperature increase. In terrestrial ecology, the basic scientific questions regarding the evolutionary ecology of insects (and other ectotherms) involved different angles from genetic to community levels. Moreover, the promotion of some biological models (i.e., Drosophila, butterfly, cricket, etc.) allowed terrestrial ecologists and evolutionarists to work on a more 'standardized' way, although the persistence of differences in the approaches. Only few studies tried to use some examples developed in terrestrial ecology to stimulate new patways in the copepodologists community. For example, Schminke (2007) used the title "entomology for the copepodologist" to compare the diversity and the life cycles of insects and copepods. Titelman et al., (2007), in their 'horizons' paper dedicated to the mating behaviour of copepods, used several well known examples in the terrestrial ecology mainly based on insects and other groups and proposed a section entitled 'ornithology for copepodologists'. These examples among others underlined the presence of gaps in our studies of copepods and that some mechanisms well studied in other terrestrial ectotherms could help to fill these gaps. One difficulty encountered when studying copepods (or other zooplanktonic groups) consists on their diversity and the complexity of their life cycles. We need thus to identify few target species in the objective to promote new emerging aquatic models then seek for the

generalization of the observed patterns. In this thesis, we focused on the estuarine copepod *Eurytemora affinis* which can be considered as a relatively well studied species and thus offers several advantages. In fact, the species is cryptic and composed by divergent genetic clades (Lee 1999) inhabiting most macrotidal estuaries in the northern hemisphere. In general, *E. affinis* can dominate the zooplankton groups in several estuaries (at least in the low salinity zones) and offers a good ecological model to compare theses aquatic habitats facing both local anthropogenic pressure (i.e., pollution, shipping, dredging, etc.) and climate changes. In fact, estuaries are very sensitive to climate change because of their position at the interface between land and ocean. One of the key questions we addressed in this thesis is:
Can we promote E. affinis as a biological model to test easily the ecological and evolutionary theories?
In order to promote this species to a good biological model, we developed in this thesis a pertinent multigenerational experimental protocol that can be simultaneously used to test several ecological theories. We particularly tried to answer these key questions, in the different following chapters:

In the first chapter, we focused in how we can develop an experimental protocol to test simultaneously reproduction and life cycle strategies in copepods (example *Eurytemora affinis*) aiming to test whether *E. affinis* is capable to cope with different environemental conditions such as temperature and/or salinity increase and food quality.

In the second chapter, we used the developed experimental protocol to verify if there is any trade-off between egg size and clutch size or egg size and reproductive effort in the copepod *E. affinis.* We also questioned about the existence of a more standardized methodology to compare both laboratory and field conditions in terms of reproductive allocation and its consequences on the life cycle traits of *E. affinis*?
We also addressed the questions of the consequences of genetic differences among different populations of *E. affinis* on the reproductive strategy of these isolated populations (case of the contrasted populations in Europe and North America)?

In the third chapter, we focused for the first time on the role that can be played by males in the reproductive strategy of *E. affinis* and consequently on the population dynamics of the species.

We particularly wanted to explore the differences between both sexes in the behaviour and the reproductive success of the *E. affinis* population. We also tried to test the hypothesis of the existence of mate selection in this copepod using the large experimental database obtained during this work.

Finally, in the fourth chapter, the experimental protocol based on careful observations of high number (several thousands) of individuals allowed to point out some abnormalities in the morphology of *E. affinis* and also studying the effects of uncommon biotic disturbances related to the presence of epibiont on copepods.

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Chapter I: Physiological Plasticity of *Eurytemora affinis*

<u>Part 1:</u> *How an estuarine copepod could face a global warming scenario? General expectations are not always true.*

<u>Authors:</u> Anissa SOUISSI[1,2,3], Sami SOUISSI[1,2,3,*], Hakim DERDAR[1,2,3], Damien BALLENGHIEN[1,2,3] and Jiang-Shiou HWANG[4]

[1] Université Lille Nord de France, F-59000 Lille, France

[2] USTL, LOG, Station Marine de Wimereux, F-62930 Wimereux, France

[3] CNRS, UMR 8187, F-62930 Wimereux, France,

[4] Institute of Marine Biology, Zooplankton and Coral Reef Laboratory, National Taiwan Ocean University, 202 Keelung, Taiwan

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Abstract

To understand the responses of individuals and populations of aquatic organisms to temperature increase, we developed a new experimental protocol to study through several generations some key life cycle parameters of the estuarine copepod *Eurytemora affinis*. The population was acclimated to a summer temperature (20°C) during 16 to 17 generations before testing the effect of a temperature increase to 24°C. The experiments were conducted under two salinities, one considered optimal (15 PSU) and one considered stressful (25PSU). We also studied the effect of a significant heat shock by transferring a well acclimated population of *E. affinis* to a low temperature (7°C) and salinity 15 to the highest experimental temperature (24°C). The effect of temperature increase was studied during 5 subsequent generations. We show here that a double stress (high temperature and high salinity) negatively affected both the growth and fecundity of *E. affinis*. The linear relationships relating prosome width and clutch size to the prosome length were highly significant. This study confirmed that the survivors in all treatments followed the general thermal rules and the copepod size always decreased when temperature increased.

We also showed that the effect of food quality on copepod growth and fecundity is much higher than the effect of salinity and/or temperature increase. *E. affinis* can rapidly cope with the shortage of food (or low food quality) after only one generation.

The results of this pilot study showed the high capacity of estuarine copepods to cope with increase of temperature and also with changes on some other environmental parameters (salinity, food). We also demonstrated that, in addition, to warming the changes of other factors, particularly food quality and salinity, could negatively affect the life cycle of *E. affinis*. The sensitivity of the population to these factors was also temperature-dependent (e.g. a greater sensitivity at the higher temperature to short term exposition to low food quality). These interactions between direct climate change and other associated modifications of the estuarine habitats of *E. affinis* should be considered in climate scenarios. The multigeneration protocol seems to be well adapted to assess the effect of any external factor on the life cycle traits of this estuarine copepod and could be generalized to any other copepod or zooplanktonic species.

Key words: Multigenerational protocol, *E. affinis*, size, clutch size, temperature, salinity.

Introduction

Temperature is often considered as one of the key external factors affecting most biological processes at individual, population and community levels (Pörtner and Knust, 2007). As a consequence, the concern of ecologists to understand the effects of warming (or climate change in general) on living organisms has been reflected by the high number of studies focusing on the effects of temperature on organisms. However, a new emerging question underlined the necessity to understand the capacity of organisms, particularly invertebrate ectotherms to face climate change (e.g. temperature increase).

Among the well documented biological models the studies of *Drosophila* spp. showed the complexity of predicting the responses of these groups to future climate change scenario (Rezende et al. 2010). The high plasticity of some invertebrates requires a careful experimental observation in order to test the existing theories related to the possible consequences of temperature increase (or climate change in general, see IPCC 2007).

Although many recent papers link physiological responses to climate change (Pörtner and Farrell 2008, Hoffmann 2010; Somero 2010), we still lack a unique standardized protocol that can generalize these emerging realtionships. We can distinguish the individual approach that allows accurate estimation of most demographic processes in addition to the conservation of the full parents-descent information. This approach could induce artifacts because individuals are isolated and thus can not reflect realistic conditions where individuals live in groups. Recently, Brown and Shine (2009) showed the importance of the number of individuals used to initiate an experiment and concluded that incubating eggs individually may modify the phenotype of the offspring. Moreover, the sensitivity of several organisms to experimental manipulation can increase the stress of individuals and induce additional biases in the estimation of the life cycle traits. We thus need a tradeoff between individually oriented experimental observations and large scale experiments (e.g., in large open systems or in the field) where the information on the history of individuals and/or their parents is often ignored.

In aquatic ecosystems, zooplankton and copepods in particular, play a key role in food webs. Most studies are based on the empirical observations of the effect of temperature (or salinity, food quality and quantity…) on life cycle traits. Temperature is one of the key external variables intensively studied. When food is not limiting, the size of adult copepods is principally controlled by temperature (Liang and Uye, 1997). However, these empirical studies and most laboratory controlled experiments were not designed to test either general ecological and physiological theories such as the temperature-size rule (Atkinson, 1994, 1995) or maternal and trans-generational effects.

Size is a key parameter in shaping the physiological as well as demographic processes (Hirst and Bunker 2003; Bunker and Hirst, 2004). The interaction between temperature and size is empirically demonstrated and some general scaling rules have been suggested (Hirst and Bunker 2003). The validation of these general rules at the species level in the framework of general ecological theories is still lacking. The high dispersion of the data, compiled from the literature based on different taxonomic levels, requires a verification of these rules at the species or genus levels.

The size structure of copepods in the field is very important for trophic interactions because predator-prey interactions are modulated by body size (Winkler and Greve, 2004). At the community level, some authors used retrospective analyses and suggested a shift in copepod community towards smaller size under the effect of climate warming (Beaugrand et al. 2003). It is difficult to associate such changes to the single effect of temperature because other modifications of both bottom-up and top-down controls could accompany global warming. The validation of these empirical observations requires an adequate experimental framework which is lacking in most cases. For other aquatic ectotherms, recent studies pointed out key mechanisms that link physiological processes at the individual level and their consequences at the population level in the context of global warming. The importance of oxygen limitation in the upper thermal limit of some fish species is one example (Pörtner and Knust, 2007). The importance of phenotypic plasticity and particularly in organisms with short life spans regarding climate change that concerns primarily increase in mean temperature (Rezende et al., 2010) and its amplitude has been underlined (Folguera et al., 2009). It seems that organisms living in highly variable environments, such as intertidal organisms (Kuo and Sanford, 2009) are better adapted to thermal stress.

The negative effect of warming can be easily demonstrated in the groups of animals having narrow thermal ranges (polar stenotherm Pörtner and Farell 2008, but see Franklin and Seebacher 2009) and low plasticity. Even if a high number of animals living in fragmented habitats or in a limited niche reached their upper thermal limit, we still have a high number of temperate eurytherm organisms that can shift their thermal tolerance and adapt to climate warming. The studies of this latter group in aquatic ecosystems in the context of climate change are still rare. However, understanding the potential of acclimatization of temperate eurythermal organisms is of primordial importance in the context of climate change studies. In fact, these species can change their thermal tolerance and favor the dominance of new phenotypes, but they can also enlarge their geographical distribution and compete with (or replace) other species unable to adapt to such changes. Somero (2010) considered that a key question is whether ecologically similar warm-adapted congeners, notably cryptic species, are capable of replacing more cold-adapted congeners that are negatively impacted by global warming. Therefore, establishing species' relative degrees of stenothermy and eurythermy in most key biological groups is necessarily step towards our understanding of ecosystem responses to global warming (Somero 2010).

Among aquatic habitats estuaries are transitional zones between land and oceans that are very sensitive to climate changes (Kimmel et al., 2006). These habitats are characterized by highly variable physical and chemical properties. Few zooplankters are adapted to live in these environments showing high physiological performances (Devreker et al., 2004) and playing key role in their food web (Kimmel et al., 2006). Among these groups *Eurytemora affinis* is a widely distributed copepod species living in most temperate estuaries, salt marshes and large Lakes in the northern hemisphere (Lee 1999; Winkler et al., 2008). It is a cryptic species group composed of a large number of populations with low levels of gene flow (except human-induced connections). We expect that this temperate eurythermal species should show a high plasticity in the face of future environmental changes but its sensitivity to warming is not well documented.

In this study we focused on the following questions:

What is the role of plasticity of *E. affinis* playing a key role in the estuarine food webs regarding climate change or, more generally, all sources of stress encountered on their habitat?

Does this species capable to adapt (or improve) to its apparent upper thermal limit observed in the field?

In this study, we focused on the extent and importance of plasticity in the acclimatization of E. affinis to environmental stress. We first developed an experimental protocol allowing experimental observation over several generations. The consideration of more than one generation is required because of the possible maternal effect on the response to external factors. In fact, the use of several generations in the experimental studies of some terrestrial ectotherms (Klok et al., 2009) is not yet generalized for copepod studies even though the maternal control of some life traits responses was confirmed (Ianora et al., 2004).

In order to study the effect of global warming on the body size and the reproduction of the calanoid copepod *E. affinis* a new multigenerational protocol was built. We selected a well studied population from the Seine estuary. This population is present all year long and shows a peak of density in late spring and early summer (Mouny and Dauvin 2002; Devreker et al., 2008). Moreover the low salinity zone of the Seine estuary, the preferred habitat of this copepod, is situated at mid- latitudes in the population distribution range in Europe. Moreover, the population of *E. affinis* in the Seine estuary can be sampled during one day by following tidal cycle (Devreker et al. 2008). During a large part of the year *E. affinis* dominates the zooplankton in this estuary (at least in the low salinity zone). The species thus represents an appropraite ecological model for extrapolating experimental results to a real field situation. Furthermore, the population of *E. affinis* in the Seine estuary does not suffer extreme climatic conditions as do more northerly populations (i.e. very low temperature and/or ice covered periods).

As in most north hemisphere estuaries, *E. affinis* population abundance decreases during summer when temperatures approach 20°C (Mouny and Dauvin 2002; Devreker et al. 2010) suggesting that 20 is the upper thermal limit of the species. In addition to temperature increase, *E. affinis* may be exposed to extreme fluctuations in salinity (Kimmel et al. 2006). We thus considered the simultaneous effect of temperature and salinity on the life cycle traits of *E. affinis*.

Souissi et al (2010) showed that the use of a multigeneration protocol allowed the track of the effect of low food quality episode leading to the possibility of observing the appearance of intersexual individuals at low temperature (7°C). In this paper we used the multigeneration protocol giving importance to the acclimation to the upper thermal limit (20°C) before testing the effect of a warming scenario of +4°C. We used *E. affinis* as an adequate aquatic model to test general ecological theories by attempting to answer the following questions:

i) What is the capacity of *E. affinis* to adapt to its thermal upper limit at optimal and stressful salinity conditions?
ii) Can we track the plasticity of *E. affinis* in both size and reproduction during several generations when applying an average (+4°C) warming scenario?

iii) What is the role of the acclimation of *E. affinis* at low (7°C) and high (20°C) temperatures before experiencing a global warming (i.e., temperature increase of 24°C)?

Materials and methods

Eurytemora affinis was collected in November 2006 in the Seine estuary (English Channel, France). They were sampled in the low salinity zone under the Tancarville bridge (49°26'N-00°16'W). A mass culture of copepods was maintained in the laboratory under controlled conditions of light, temperature and food, according to the protocol detailed in Souissi et al. (2010).

Before starting the experiments, a common copepod batch culture was acclimated to certain laboratory conditions (12h light /12h dark, salinity 15 PSU and temperature 15°C) during few generations to remove the historical imprint of maternal and trans-generational effects.

After x generations, the batch culture was split in two to start the multigenerational experience (the F0 generation). The first half was initially acclimated to 10°C and a salinity of15 PSU and then acclimated to 7°C. The second half was acclimated to 20°C and salinity 15 PSU. At 20°C, from the culture realised at salinity 15 a second culture starting one generation later was also initiated but at higher salinity of 25. This higher salinity is stressful for *E. affinis* from the Seine estuary to its development, survival and reproduction (Devreker et al., 2007; 2009). A set of three regimes was established to test the multigenerational effect of different conditions. We used 40 ovigerous females to start each regime culture to avoid any inbreeding risk. The first regime (Rg1) is a copepod culture raised at 7°C and 15 PSU (T7S15), the second regime (Rg2) is raised at 20°C and 15 PSU (T20S15) and the last regime (Rg3), which started one generation later, is raised at 20°C and 25 PSU (T20S25). These three regimes were followed during several generations for the pre-experimental period, corresponding to a period for acclimation before starting the experiment. In the Rg1 this period extended from F1 to F2, in Rg2 it's from F1 to F10 and in Rg3 the acclimation was from F1 to F9 (Fig. 1). The shortness of the acclimation period at 7°C compared to 20°C is explained by the length of the generation time. The mean copepod developmental time is 42 days at 7°C and 12 days at 20°C.

We consider high number of generations to better acclimate the population to the supposed upper thermal limit conditions (according to our *in situ* observations). Moreover, to remove any potential effect linked to genetic heterogeneity in the starting batch the high salinity treatment T20S25 was initiated from the first generation at T20S15 (this explains the lag in the number of generations between salinity treatments). After acclimation, the study of the effects of different conditions began. Rg1 was carried out during 4 successive generations in T7S15 and 8 generations in Rg2 and Rg3 under T20S15 and T20S25 respectively (Fig. 1). The experimental protocol of the generation's follow-up is well detailed in Souissi et al (2010). These *E. affinis* culture regimes were fed ad lib once a day with a non-limiting food algae (*Rodhomonas marina* algae at its exponential phase of growth) to avoid any potential excess of food that can alter the feeding habits. Nevertheless, constraints on algal growth did occur providing the opportunity to test the effect of a short term episode of low food quality (Fig 1). This low food quality episode coincided with the development of F4 at T7S15 (T7S15(F)), F16 at T20S15 (T20S15(F)) and F15 at T20S25 (T20S25(F)). After the acclimation period, all regimes were simultaneously transferred to 24°C and followed during 5 more generations (Fig. 1).

From each generation of each condition, 20 to 40 adult ovigerous females and males were sorted, the prosome length and width were then measured under an inverted microscope (OLYMPUS IX71) and using the image analysis software package Image J 1.41 (Rasband, 1997-2008) as described in Souissi et al. (2010). The fecundity of females was also estimated by counting eggs in each female's ovisac (clutch size). The protocol was designed to use the first clutch for all generations and thus avoid any age effect that was observed in the reproduction of this species (Deverker et al., 2009).

To test the relationships between all measured variables we used the Pearson correlation coefficient. When relationships are statistically significant a linear correlation between variables was applied. Moreover, the cumulative distributions of the observed prosome lengths of females and males in each experimental condition were fitted by using a Normal (Gaussian) cumulative probability density function. All statistical analyses were performed with Matlab Software. The curve fittng toolbox was used to estimate the fitted parameters, confidence intervals and goodness of fit.

Results

Multi-generational responses of the body size to the experimental conditions

The general observation of the fluctuation in the body length and width through all the experimental conditions revealed a clear sexual dimorphism with females being the bigger than males. This dimorphism is more pronounced in the width size (Fig. 2). Each population in all conditions showed an intergenerational variability in the body size and this is due to the intra-population regulation density (Chapter I, Part 2).

Testing the low food quality effect

In all conditions, the low food quality supply event induced an immediate decline in the body size in the following generation, but this episode was less intense at the lowest temperature condition T7S15 (Fig. 2A). The decline in the size was of 3.53%, while at T20S15 and T20S25 the size decrease was 5.67% and 9.72% respectively (Fig. 2B,C). We noticed here the effect of the additional stressful salinity of 25 PSU. The same pattern was also observed for body width with a decrease of 6.12% at T7S15, 4.82% at T20S15 and 12.41% T20S25 (Fig. 2D,E,F).

The low food quality episode lasted for only a few days and its effect did not exceed one generation (Souissi et al., 2010). Indeed, in all conditions, body size was recovered and at times surpassed in the next generation (table 1). Males also showed the same reaction to the low food quality episode (Fig. 2) but experienced generally lower proportion of loss than for females (see Table 1). The differences between the sexes were also observed in their capacity to rapidly overcome these negative conditions. However at the lowest temperature the most significant decrease in prosome length of females was observed two generations later, leading to suggest a possible delayed maternal effect.

Testing the effect of a global warming scenario.

Contrary to the effect of low food quality, the transfer to 24°C from T7S15 induced the highest effect on male and female body size with respectively a decrease of 16.78% and 18.14% in the length and 18.99% and 16.00% in the width during the first generation. The transfer from the other conditions also induced a decline in both male and female sizes during the first generation but with almost half of the amplitude (Table1).

In all conditions, *E. affinis* survived at 24°C and developed during 5 successive generations until the end of the experiment. However, the patterns of male and female sizes varied betweenconditions andgenerations (for the same condition).

After the transfer to the higher temperature a slight recovery in body size was observed through the generations for the experiment (T7S15). In fact, we observed for the female a recover of 10.35% in body length and 13.94% in body width. In addition, males recuperated 5.77% in length and 7.74% in width. For the other set of experiments (T20S15) and (T20S25) the copepods presented lower but statistically significant percentages of recovery compared to (T7S15) (Table 1). In spite of this recovery, the size at the last generation of the transfer to 24°C still very far from the size at the starting point generation and significantly different, in all experimental regimes (Table1). For example, the prosome length of the female in the last generation at T24(7)S15 was 12.86% lower than the initial size (F3). Even if it recovered after experiencing a very strong heat shock, the same pattern is also observed at T24S15 and T24S25 showing respectively 3.84% and 7.8% lower sizes than F11 and F10, respectively.

The transfer of copepods to 24°C did not result in the same size in the first generation for all individuals from the different experiments.The highest value was for individuals from 7°C, even though it is the most stressful transfer with the largest decline in size. Also the recovery at the last generation gave the greatest increase in the size of individuals from the (T7S15) condition. These individuals appeared to catch up to their initial sizes, resulting in the highest values after 5 generations at 24°C compared to the copepods acclimated to the high temperature of 20°C (Fig. 2, Table1).

In spite of these differences in the response of the body size to either low food quality or high temperature, the allometry of the body size in *E. affinis* individuals followed the same trend and showed always a difference between sexes (Fig. 3). In fact when we gathered all the data in one graph to test the relationship between the prosome length and width, we found that for all confounded regimes, the body size showed two clear different trends for the male and the female. These two morphological traits have a positive significant correlation (the female: $y=0.351x+22.82$, $R^2= 0.85$; $p<0.0001$, male: $y=0.305x-1.374$, $R^2= 0.9$ $p<0.001$) with almost a parallel slope for the female and male patterns. These slopes were 0.351±0.053 and 0.305± 0.037 respectively (Fig. 3).

The cumulative probability distributions of prosome length in all experimental treatments were computed and fitted for females (Fig. 4A) and males (Fig. 4B). All distributions were symmetrical and consequently well fitted by a Gaussian distribution (all values of $R^2 > 0.99$, see Table 2). The effect of all experimental conditions on prosome length was observed on both μ and σ in both sexes (Table 2). The values of μ were very close to the observed means of each experimental condition (data not shown here) and confirmed the negative effects of low food conditions, temperature and/or salinity increase on prosome length. The analysis of the individual variability in growth (i.e., prosome length) through the parameter σ confirmed the higher plasticity of females compared to males (Table 2). The highest value of σ observed at T7S15 could be explained by the possible maternal effect observed in the last generation F6 (see Fig. 2A). For both sexes and in all conditions, the low food event reduced significantly both values μ and σ (Table 2), whereas the heat treatment decreased σ for females and increased it for males (except when salinity was high).

Multi-generational responses of the clutch size to the experimental factors

The comparison of the mean clutch size of the ovigerous females, in the different experiments, showed that copepods at low temperature were more affected in their fecundity by the thermal stress than by the food stress, in contrast to the copepods at the 20°C conditions. The latter suffered few in their clutch size after the transfer to 24°C but the highest decrease was registered at the low food quality event and this response was stronger at the high salinity 25 (Fig 5A). For each set of experiments the mean clutch sizes were significantly different (t-test, $p<0.001$) except for T7S15 and T7S15(F).

The mean clutch size presented was multigenerational and did not take into consideration the generation time (development time from egg to ovigerous female). If we consider that by normalizing the clutch size we obtain the daily reproductive proportions for all conditions (Fig. 5B). This standardization reveals that the clutch size at the lower temperature was in reality the lowest in all conditions and gave the same pattern for the food. After the transfer to 24°C the copepods increased their normalized reproduction. For the other conditions the clutch size decrease was not very high compared to the response to the low food quality.

The relationship between the clutch size and the body length in all conditions followed the same pattern giving a positive significant trend ($y=0.12x - 75.47$, $R^2 = 0.88$, $p<0.0001$) (Fig. 6) with higher variation in the clutch size than in the prosome length particularly at the low temperature.

Discussion

In order to test fundamental ecological and evolutionary theories empirical evidence from experimental conditions are still needed. It is difficult to extrapolate some conclusions from one biological model to another one. For copepods, an intensive work already confirmed the key role of temperature in all demographic processes (Huntley and Lopez, 1992; Bunker and Hirst 2004). However in the field conditions the effect of temperature is always combined with those induced by other external factors such as food availability, salinity, pollution, predators' etc. We can rely on empirical studies in the field only in few examples when food is not limiting. For example Liang and Uye (1997) linked the growth, the mortality and the production of the key copepods in the Inlet Sea of Japan to temperature. We can see, in all these examples, that copepods clearly follow the temperature size rule, with the large individuals observed during cold season and the smallest individuals observed during summer. Except parasitic copepods that may transgress the temperature-size rule (TSR), it seems that free living copepods belong to the high number of taxa that follow this rule (Atkinson 1994, 1995).

In the context of climate warming, the confirmation of the TSR in the selected copepod species is not the ultimate objective by itself, but the understanding of their plasticity and sensitivity to climate change is needed. In this context, both terrestrial and aquatic ecologists face the same scientific challenge of the comprehension of mechanisms at the scale of the individual, the population and the community.

Pörtner and Farrel (2008) suggested to link between physiology at individual scale and fitness at the population level. Other studies based on more classical terrestrial models (i.e. Drosophila) showed that the capacity of a population to react to an increase of temperature is rather complex (Rezende et al. 2010). Some studies used multi-generation protocols and confirmed the importance of designing adequate protocols. In copepods, the multi-generation protocols only concerns some ecotoxicological applications (Lee et al., 2008) or the effect of high CO_2 (Kurihara and Ishimatsu, 2008) and the total number of generations never exceeded 3. On the other hand, some studies performed a high number of generations in *Drosophila* (Klok et al., 2009) and butterfly (Beldade et al., 2002). The objectives of each work were different from the previous studies and of course from technical point of view the experimental protocol should be adapted to each biological model.

In this study, we proposed a new multigenerational protocol to test our working hypotheses regarding climate change scenario on an estuarine copepod. The study was designed to focus on more general conclusions and to try to enrich the ecological debate around the climate change issue rather than be specific to such biological model.

The copepod *Eurytemora affinis* can be considered as an appropriate model to be used in this ecological context because of the following reasons:

i) Wide geographical distribution and high potential to invade freshwater ecosystems (Lee 1999).

ii) In estuaries, the populations are restricted to the low salinity zone and thus are isolated from each other and can face high variability of environmental factors at different scales (Kimmel et al., 2006; Devreker et al., 2008)

iii) The species is eurytherm; this means that it has a high plasticity. But in the field it is limited to cold season (despite its presence all the year in most estuaries). We can question about the real thermal limit of this species? Why its distribution is so limited in the south?

Our experimental protocol was designed to first acclimate copepods during several generations to cold and high temperature. We consider 20°C as the upper thermal limit because in the field the population drops during the summer season with such temperature (Mouny and Dauvin 2002; Devreker et al. 2010). We found that *Eurytemora affinis* (as most free living copepods, see Atkinson 1994) followed the general temperature size rule. Even if the mechanisms of such general empirical rule in ectotherms are still illusive, we can admit that the suggested hypotheses may apply to *E. affinis* (Walters and Hassal, 2006).

In *E. affinis,* we confirmed that at low temperature large females produced larger clutches which offers a considerable advantage to *E. affinis* in the Seine estuary (and in other habitats in general). In fact, after the over-wintering season, the surviving individuals at low temperature will tend to improve the fitness of their offspring by increasing their clutch size. This generation will develop during the seasonal temperature increase from March to May and engender a high recruitment. For that reason the seasonal maximum abundance of *E. affinis* coincides with this favourable temporal window (Devreker et al., 2010). However, if the critical period at low temperature did not allow females to reach high size and/or produce large clutches, the annual peak logically observed during May-June can be seriously affected.

This situation was encountered in 2005 when *E. affinis* over-wintering stock (adult females) experienced a strong negative thermal anomaly due to regional climate (Gomez and Souissi 2008). The consequence of this strong stress resulted in a very low abundance in late spring population (i.e. May 2005 in Devreker et al. 2010) compared to the densities reached by *E. affinis* in the same period in 2004 (Devreker et al. 2008).

Ovigerous females of *E. affinis*, in the Seine estuary were observed during all the year, this means that the reproductive activity never stops. The latitudinal position of the Seine estuary offers a temperature range favourable for its growth and development. But in all years the density of *E. affinis* dropped during summer when temperature approached 20°C. We can suppose that this temperature is close to the upper limit of the species. According these reasons we selected the temperature 20°C to test the possible effect of acclimation to the upper thermal limit. The first conclusion is that 20°C seemed to be physiologically adequate temperature for *E. affinis* development. This means that may be other factors such as the presence of predators during summer especially mysids (Winkler and Greve 2004) could explain the drop of *E. affinis* population. *E. affinis* can develop in a high range of temperatures as indicated in previous studies (Katona 1970; Bradley 1978; Bradley & Ketzner 1982). Katona (1970) cultured *E. affinis* in the range of temperature from 2°C to 23.5°C. He observed that the temperature increase from 2°C to 20°C engendered a decrease in the total length of *E. affinis* males and females about 30% and 42% respectively. The differences between male and female sizes were also confirmed in this study. The same pattern was observed by Ban (1994) who cultured *E. affinis* at three temperatures 10°C, 15°C and 20°C. Moreover, Ban (1994) showed that the effect of food concentration is very important for the development and the growth of *E. affinis*. We showed in our study that a short period of low food quality affected negatively the population of *E. affinis* but more proportionally at 20°C compared to 7°C. Souissi et al. (2010) (Chapter IV, Part 1) showed that the effect of this low food event at 7°C was subtile and engendered the appearance of intersexual individuals. It is, thus, important to consider the combined effects of temperature and food availability (quantity and quality) in the context of climate change effects on copepods and aquatic ecosystems in general.

After few generations of acclimation to either low or high temperature, *E. affinis* was viable in a higher temperature of 24°C (+4°C scenario). In the current climate conditions, *E. affinis* may not encounter such temperature in the low salinity zone of the Seine estuary (Devreker et al., 2010). However we noticed that in the laboratory this temperature is not lethal. The presence of *E. affinis* population in higher summer temperature is recorded, for example, in the Chesapeake Bay (Kimmel et al., 2006). But we should be careful when making such comparisons, because *E. affinis* is a species complex composed by genetically divergent populations. The reaction norms for this species were studied regarding salinity variation (Lee et al., 2007) but not regarding temperature variation. It is possible that the populations situated in the most southern latitudes (i.e. Chesapeake Bay) could show better adaptation to higher temperatures. But this hypothesis needs to be verified in future experimental work.

The population of *E. affinis* from the Seine estuary showing an apparent preference to low salinity (Devreker et al. 2008) which is most likely associated to its adaptation to local hydrodynamics was not strongly affected by an additional stress due to high salinity treatment. The high capacity of osmoregulation of this species allowed maintaining a good performance at salinity 25 under two high temperatures (20 and 24°C). The performance was lower for the fecundity at higher salinity but the effect still less intense than the food effect. Our experimental results confirmed the existence of a real difference between the empirical field observations of the optimal (i.e., upper limit) temperature or salinity and the real physiological potential of the species. We demonstrated clearly that *E. affinis* under non limiting food conditions can survive and develop well at 20°C and also 24°C. This means that the absence of a maximum abundance in summer is due to other reasons. In fact warm season was more favourable to the development of the broadcast spawner group of genus *Acartia*. Our field observations did not confirm a real overlapping between the habitats of *E. affinis* (preferring low salinities) and *Acartia* sp. (preferring higher salinity) leading to conclude that the seasonality of each species is related to their differences on life cycle strategies. Furthermore, the main predator of *E. affinis* in the low salinity zone of the Seine estuary is the mysid *Neomysis integer* (Mouny and Dauvin 2002; David et al. 2006). The risk of high predation at this season could explain the apparent pattern of *E. affinis* with a maximum of abundance observed below 20°C. Other factors such as the food availability and its quality during summer could affect negatively *E. affinis*. Finally, the relative shortness of the summer season (Devreker et al. 2010) does not allow the acclimatization of *E. affinis* population to local conditions at least in the Seine estuary. Generally, in European populations of *E. affinis*, the southern distribution is limited to the Gironde estuary (excepting some records of low number of individuals in other estuaries). This means that *E. affinis* could have some thermal limitations that reduce its competitiveness compared to other copepods species such as *Acartia tonsa* (David et al., 2007).

Overall the mechanisms leading to explain the high performance of *E. affinis* from the Seine estuary at high temperature it is worth noting that even after long acclimation to a low temperature, a severe heat shock (transfer from 7°C directly to 24°C) was not lethal for this population. We suppose that the low temperature strategy giving large females producing large clutches contributed to this resistance. Moreover, it seemed that the size of females was heritable. In fact, individuals originated from 7°C and reared at 24°C reached the highest size at 24°C even after 5 generations. The maternal control could not explain the trans-generation persistence of this pattern. It is possible that the size is heritable in *E. affinis* and this should be addressed in future experimental studies.

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Tables

Table 1. Summary of the relative differences in body size of males and females of *E. affinis* in the key generations considered during the study. PL: Prosome length, PW: Prosome width

		Experimental regimes	**(T7S15)$_s$**		**(T20S15)$_s$**		**(T20S25)$_s$**	
		sexe	♀	♂	♀	♂	♀	♂
Low food quality	**PL**	Loss (%)	-3.53	-3.77	-5.67	-6.43	-9.72	-4.96
		Recover (%)	2.28	1.25	10.97	8.39	15.42	9.09
	PW	Loss (%)	-6.12	-2.01	-4.82	-8.05	-12.41	-4.30
		Recover (%	4.75	2.03	17.91	10.66	20.07	10.18
Transfer to 24°C	**PL**	Loss (%)	-18.14	-16.78	-8.20	7.45	-8.98	6.61
		Recover (%) compared to the 1st transfer generation	10.35	5.77	1.07	0.58	3.04	1.04
		(%) difference with the starting point generation	-12.86	-15.30	-3.84	-5.48	-7.80	-6.55
	PW	Loss (%)	-16.00	-18.99	-10.22	-3.91	-6.58	-4.45
		Recover (%) compared to the 1st transfer generation	13.94	7.74	0.90	-1.32	1.78	3.52
		(%) difference with the starting point generation	-10.15	-14.48	-0.72	-7.05	-5.13	-7.92

Table 2. Values of the fitted parameters of the Normal cumulative density function for the prosome length and their associated statistics. μ: the mean of the Normal distribution (values are given with 95% of confidence intervals), σ: the standard deviation of the Normal distribution (values are given with 95% of confidence intervals), n: the number of individuals, R²: coefficient of determination, SSE= the sum of squares due to errors, RMSE= the root mean squared errors. A value closer to 0 indicates a better fit.

Condition	μ	σ	n	R²	SSE	RMSE
Females						
T07S15	991.1 ± 4.05	65.03 ± 5.70	121	0.9952	0.0161	0.0284
T07S15F	994.2 ± 2.35	38.23 ± 3.31	40	0.9976	0.0093	0.0215
T24(7)S15	854.4 ± 1.7	46.45 ± 2.38	148	0.9989	0.0039	0.0140
T20S15	896.3 ± 0.95	35.43 ± 1.35	196	0.9996	0.0017	0.0091
T20S15F	825 ± 0.65	28.81 ± 0.94	40	0.9997	0.00099	0.0071
T24S15	850.8 ± 0.40	31.55 ±0.54	200	0.9999	0.00029	0.0038
T20S25	879.7 ± 1.60	34.25 ± 2.23	167	0.9989	0.0047	0.0153
T20S25F	785.3 1.45	28.95 ± 2.07	23	0.9980	0.0048	0.0155
T24S25	838.6 1.00	32.01 ± 1.38	158	0.9995	0.0019	0.0098
Males						
T07S15	956.5 ± 1.90	33.64 ± 2.68	61	0.9983	0.0081	0.0187
T07S15F	940.1 ± 1.85	35.47 ± 2.63	20	0.9985	0.0074	0.0179
T24(7)S15	799.5 ± 1.50	37.32 ± 2.14	100	0.9989	0.0046	0.0142
T20S15	854.7 ± 0.45	21.52 ± 0.62	120	0.9999	0.00067	0.0054
T20S15F	790 ± 0.90	15.83 ± 1.21	20	0.9992	0.0037	0.0127
T24S15	811.7 ± 0.45	28.21 ± 0.69	100	0.9999	0.00063	0.0052
T20S25	828 ± 0.75	29.27 ± 1.06	120	0.9997	0.0015	0.0079
T20S25F	772.4 ± 0.95	16.42 ± 1.33	20	0.9990	0.0042	0.0135
T24S25	793.7 ± 0.80	24.89 ± 1.08	100	0.9996	0.0018	0.0088

Figures

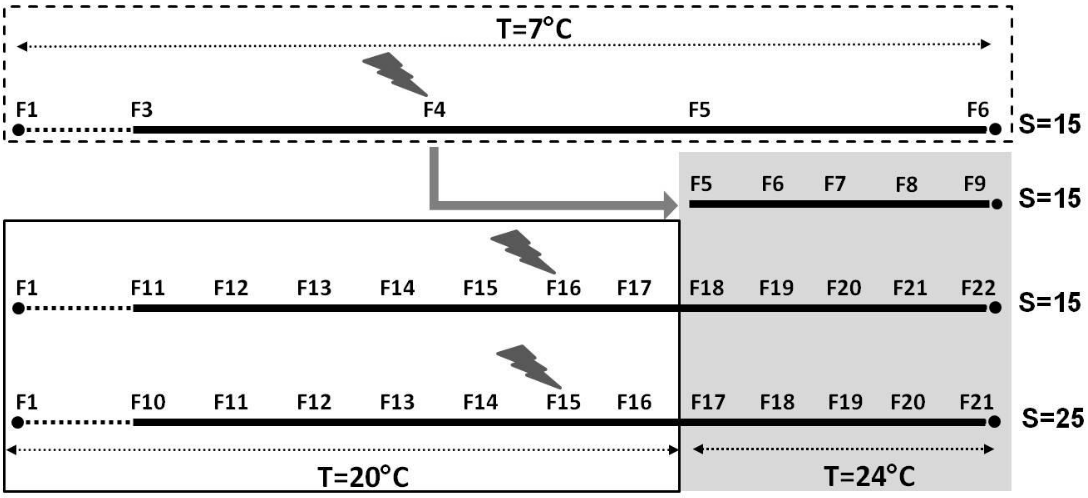

Figure1. Schematic illustration of the different sets of experimental culture conditions using the multi-generation protocol. The dashed double arrows lines and the three boxes show the different temperature conditions used in the multi-generation experiments. The thick lines with label shows the number of generations followed and used in this work, the starting dashed part of these lines present the acclimation period of the copepods before starting the experiments. The salinities shown by the labels: S=15 and S=25 are used in the experiments at two temperatures 20°C and 24°C. The grey arrow illustrates the transfer of the copepod culture from 7°C to 24°C from the generation F4. The dashed line rectangle encloses the experiment conducted at 7°C, the continuous line rectangle contain the set of experiments conducted at 20°C and the grey rectangle represents the experiments done at 24°C. The grey pointers specify the low food quality supply events to each concerned copepod culture.

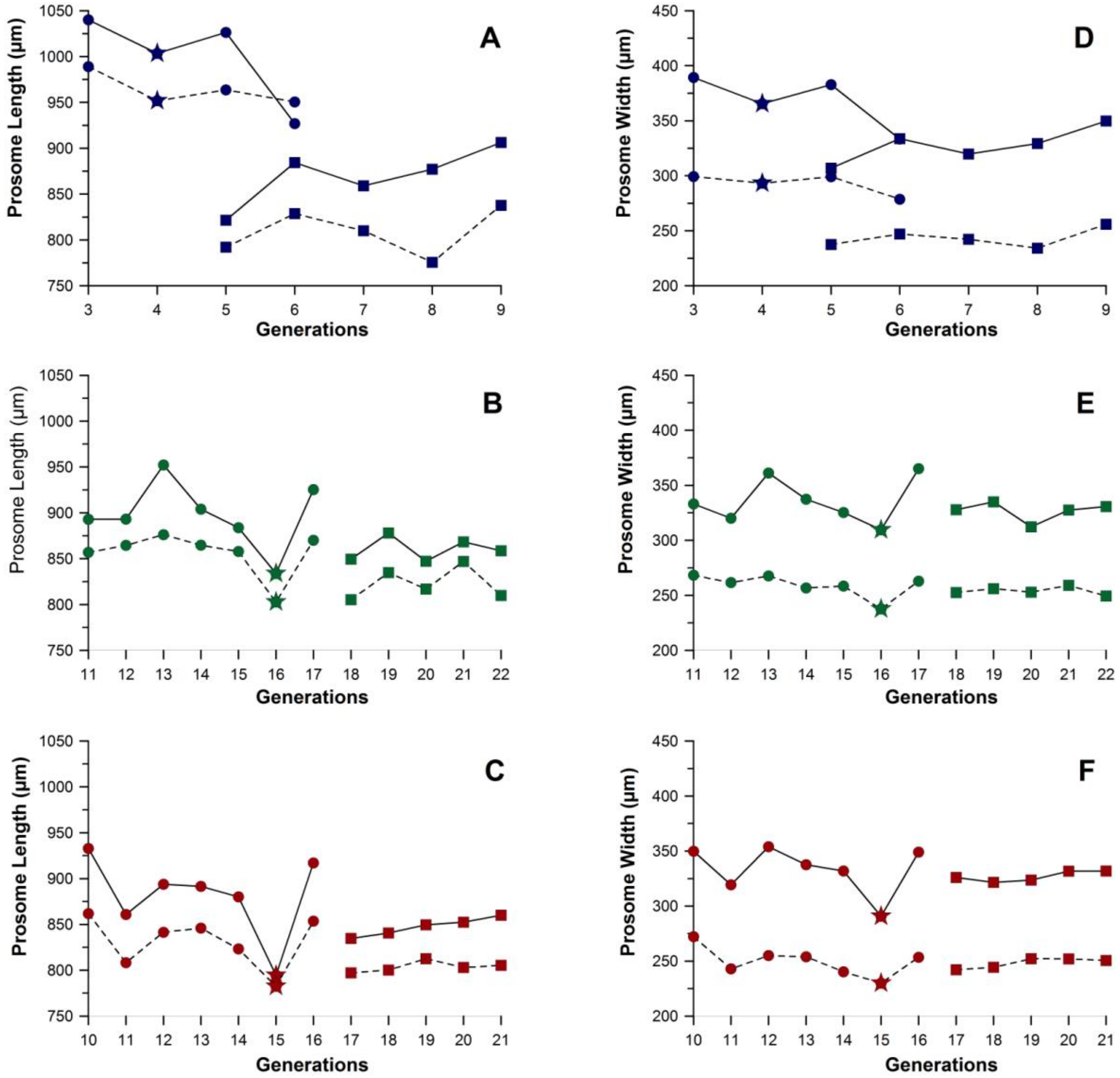

Figure 2. Mean prosome length (A, B, C) and prosome width (D, E, F) as a function of the different generations in the different experimental conditions. Circle labels indicate data from the starting experimental conditions, star labels indicate data from the low quality food event, and square label indicate the data after the transfer to 24°C in each experimental condition.

The blue color designate the data from the T7S15 condition, the green color designate the data from the T20S15 condition and the red color designates the data from the T20S25 condition. The continuous line specifies the data of females copepod and the dashed line indicates data for male copepods

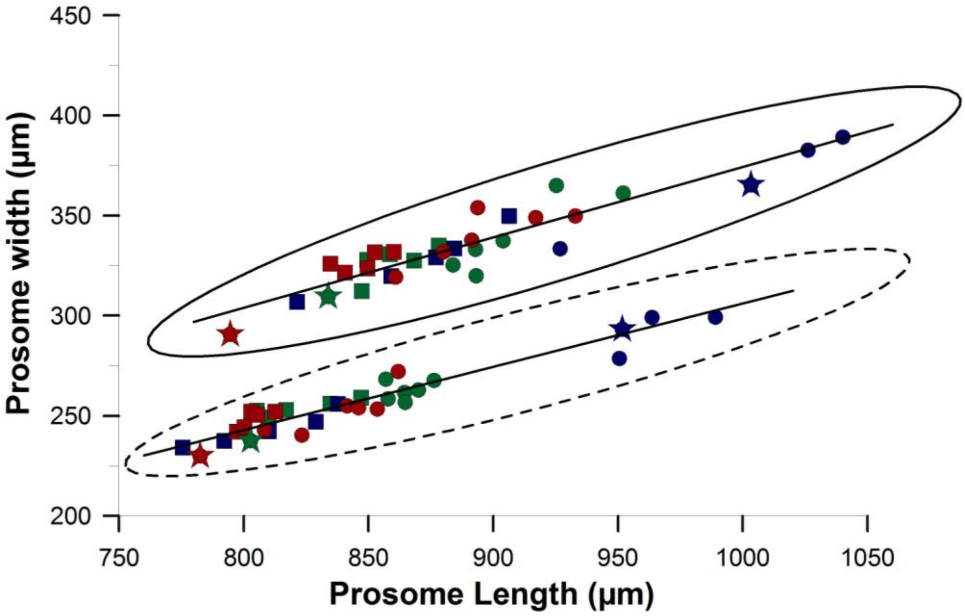

Figure 3. Correlation between prosome length and width of *Eurytemora affinis*'s males and females in all experimental conditions. The data correspond to the average values in each generation as shown in figure 2. The continuous line ellipse separates the female's copepod plotted data and the dashed line ellipsoid male's copepod plotted data. The black lines represent the linear fit for females ($y=0.35x+22.82$, $R^2= 0.86$) and for males ($y=0.3055x+1.3744$, $R^2=0.9014$), respectively.

Label colors and shapes are the same as in the previous figure (2) and indicate the different experimental conditions.

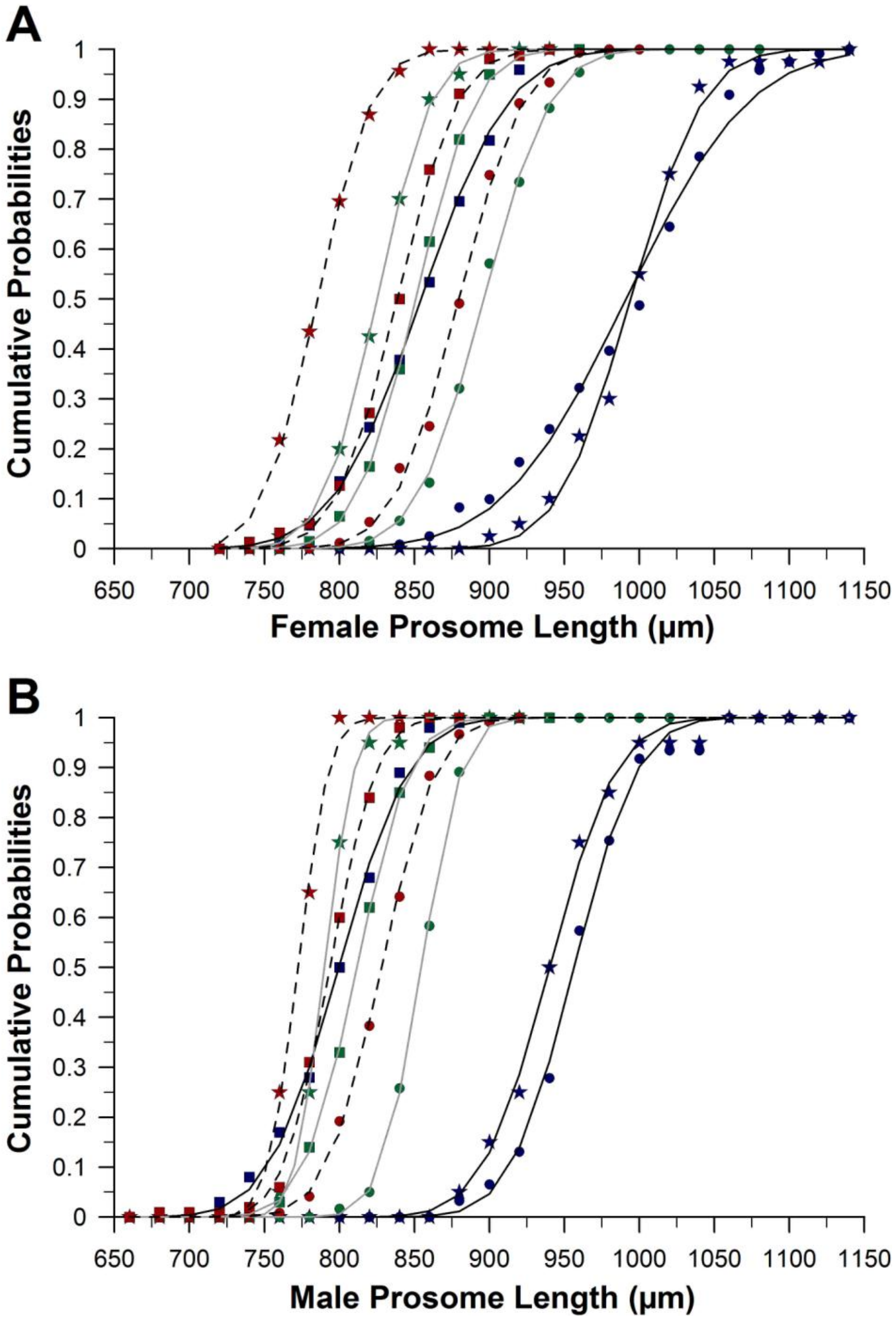

Figure 4. Distribution of the cumulative probabilities of the prosome length over the different experimental conditions. Label colors and shapes are the same as in the previous figure (2) and indicate the different experimental conditions. The observed data were fitted by a normal cumulative probability density function (see table 2). The different fitting lines show the different set of experiments: the black line point out the data from the T7S15 condition, the grey line indicates data from the T20S15 condition and the dashed line shows the data from the T20S25 condition.

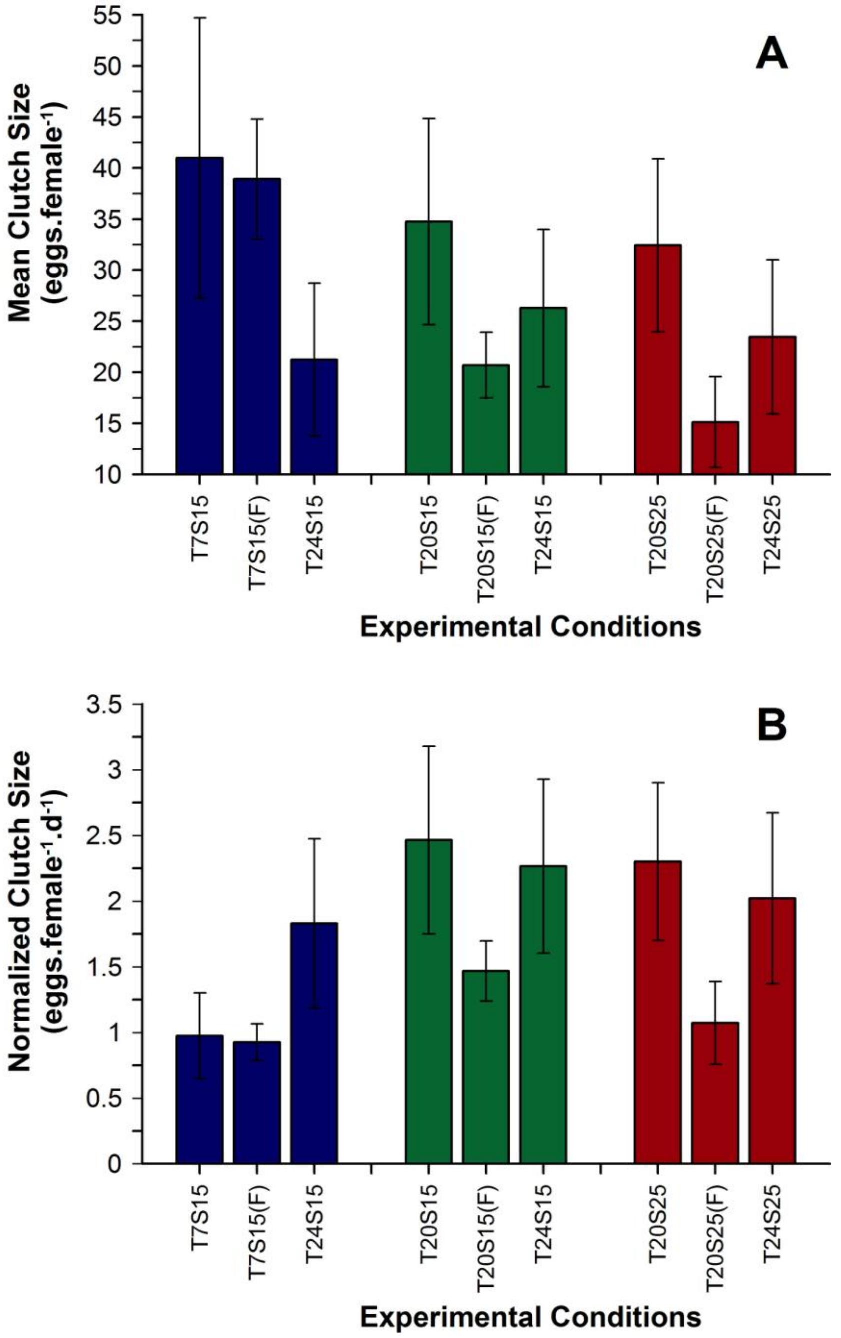

Figure 5. Clutch sizes of *Eurytemora affinis* at the different experimental conditions. A: the mean clutch size of *E. affinis* females at each experimental condition, B: the normalized clutch size of *E. affinis* females per day at each experimental condition. The color labels of the bar charts separate the initial conditions: the blue shows the data from the condition T7S15, the green is for the data from the condition T20S15 and the red color is for the T20S25 condition. The vertical bars present the standard deviation.

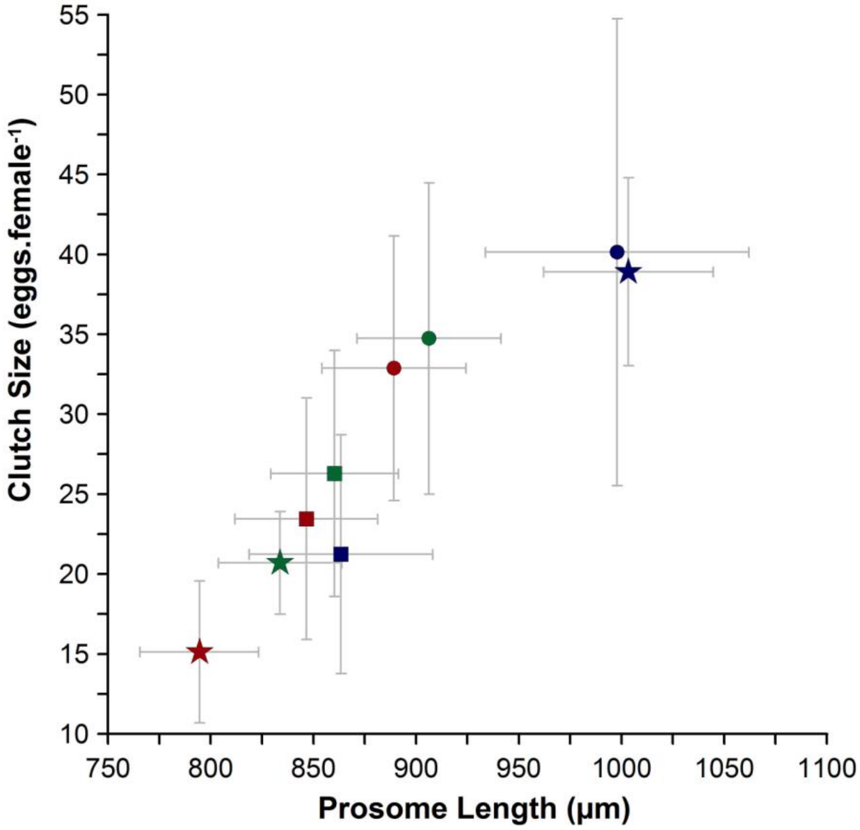

Figure 6. Relationship between the mean female's prosome length and clutch size at the different experimental conditions. The labels used to design the data from the different conditions are the same as in Figure 2. Error bars show the standard deviation.

Part 2: ***Multigenerational responses of life cycle traits to temperature increase in three genetically divergent populations of the copepod Eurytemora affinis***

Authors : Anissa SOUISSI[1,2,3], Sami SOUISSI[1,2,3,*], David DEVREKER[1,2,3] and Jiang-Shiou HWANG[4]

[1]Université Lille Nord de France, F-59000 Lille, France

[2]USTL, LOG, Station Marine de Wimereux, F-62930 Wimereux, France

[3]CNRS, UMR 8187, F-62930 Wimereux, France,

[4]Institute of Marine Biology, Zooplankton and Coral Reef Laboratory, National Taiwan Ocean University, 202 Keelung, Taiwan

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Abstract

We studied experimentally the effect of a warming scenario on the life cycle traits of the estuarine copepod *Eurytemora affinis*. We developed a new multigenerational experimental protocol and tested the hypothesis of life-cycle plasticity of estuarine copepods by using three populations of *E. affinis* from different European and North American estuaries. We demonstrated that after 16 generations of acclimation to 20°C all populations of *E. affinis* were capable of coping with a temperature increase of 4°C. At this *in situ* upper thermal limit (20°C) a negative density dependent mechanism on female's growth was shown. This mechanism allowed regulating the population in absence of any external perturbation (e.g., predators). When the three population cultures encountered a short term a low food quality episode, they were more sensitive at higher temperature than at lower temperature (7°C). Moreover, the oldest copepod culture originated from the St Lawrence estuary (North America) showed the highest sensitivity to each stressful event (low food quality episode and temperature increase) leading to suggest that laboratory selection reduced the resilience capacity of this population. The mortality rate increased with temperature but remained low in our experimental conditions. Most life cycle traits showed the same pattern in their responses to temperature, whatever the origin of the population. Only sex-ratio in the Seine estuary was different from the value observed in Gironde and St Lawrence populations. It seems that the male biased sex-ratio of the population of *E. affinis* from the Seine estuary is an intrinsic characteristic of this population. The genetic origin of the sex-ratio was confirmed but the advantages of such male biased sex-ratio in the Seine estuary are still not elucidated.

Key words: *Eurytemora affinis*, Generation, Temperature, Trade-off, Fitness

Introduction

The different forms of biodiversity (genetic, morphological, functional, etc.) exhibit complex interactions and play a determinant role in shaping ecosystem responses to external forcing acting at different scales. Determining the relative importance of these factors is both difficult and urgent, particularly nowadays when ecosystem health appears under threat from climate change, pollution and over-exploitation. A current challenge for scientists is to understand how different scales of organisation (individual, population, community, and ecosystem) may respond to future global changes. Various scenarios of climate change on a global scale are documented in the recent IPCC assessment report (2007). In addition to general trends of most climatic variables (i.e. precipitation, temperature) the occurrence of extreme events is of major concern. For example, positive or negative thermal anomalies may affect individual species occurrence and consequently ecosystem functioning (Gómez and Souissi, 2007; 2008). However the exact interaction among the complexities of biological responses to climate change requires experimental work to understand the exact mechanisms contributing to the evolution of aquatic and terrestrial ecosystems in a future world.

Temperature, acting at different scales, is one of the key factors affecting the development of ectotherms,. Despite the high number of publications dealing with the interaction between temperature and various biological processes, a unifying theory of thermal adaptation is still lacking (Angilletta et al. 2006). Thanks to intensive multidisciplinary studies on some biological models, the complexity of the interaction between environmental temperature and population dynamics involving processes from genes to the organism has been identified (Bennett et al., 1990; Cooper et al., 2001). For example, due to their short generation time and the ease of measuring their fitness, the bacterium *Escherichia coli* has been intensively used to study the effect of thermal adaptation on population growth (Cooper et al., 2001). However the extrapolation of these conclusions to other biological groups is not always possible because life cycle characteristics and evolutionary potential tend to be taxanomically specific. We thus need to promote new biological models in different ecosystems to study the phenomenon of thermal adaptation.

Among aquatic organisms, zooplanktonic species are good candidates to develop different biological indicators. By their pivotal role linking primary producers and upper trophic levels, variations in the population size of zooplankton may affect the productivity of aquatic ecosystems. Modifications in the structure and timing of seasonal peaks in plankton communities may lead to varying degrees of mismatch between trophic levels and functional groups, and therefore alter food-web structures (Beaugrand et al., 2003; Winder and Schindler 2004). Several aquatic ecosystems including lakes, estuaries and wetlands are discontinuous and their zooplankton communities evolved in isolation with few opportunities for genetic exchange. The consequences of geographical isolation and/or genetic differences among zooplanktonic species on ecosystem functioning is a crucial question at present poorly documented in the literature. In particular, estuarine copepods, particularly those few euryhaline species capable of living in highly variable environments, are composed of genetically divergent clades. Caudill & Bucklin (2004) found highly divergent clades of an estuarine copepod, *Acartia tonsa*, among estuaries of the northwest Atlantic coast. Similarly, *Eurytemora affinis* complex is composed of genetically distinct clades, representing divergent evolutionary histories (Lee 1999; Lee & Frost 2002).

Crustacean zooplanktonic species posses complex life cycles generally composed by a high number of developmental stages, including diapause, and their reproductive strategies, are difficult to study. This helps to explain the low number of experimental studies comparing physiological adaptations and environmental tolerance of different zooplankton populations. Mitchell and Lampert (2000) maintained, under standardized cultures, clones from 8 populations of *Daphnia magna* across Europe. They showed temperature adaptation responses in *D. Magna* through the variation in the shape of the temperature reaction norms when it varied between 17°C and 29°C. In the USA, Lonsdale and Levinton (1986) studied under identical experimental conditions the harpacticoid copepod *Scottolana canadensis*, taken from a broad range of latitudes. They showed that when temperature increased from 15°C to 25°C, the growth rate of only the southern-most population of copepods continue to increase. More recent studies on the free spawning calanoid copepods of the genus *Acartia* showed that the temperature responses of two allopatric populations of *A. clausi* from northern and southern Europe were different, particularly at high temperature (Leandro et al. 2006). Under the same experimental conditions, Drillet et al. (2008) showed significant differences in the vital rates of four *Acartia tonsa* cultures originated from different trans-Atlantic locations. However, these studies did not allow testing the general ecological questions on the life cycle plasticity of copepods in a context of climate change. We need to develop new experimental protocols to assess general theories in the regulation of life cycles and the link between individual and population processes.

The copepod *Eurytemora affinis* plays a key role in the food webs of temperate estuaries. This species complex is composed of genetically divergent clades capable of invading freshwaters (Lee, 1999). Early studies of temperature adaptation in the population of *E. affinis* from the Chesapeake Bay, where it may encounter a wide range of temperatures (0°C to 30°C) demonstrated that females showed higher temperature tolerance and acclimation than males (Bradley 1978). Bradley and Ketzner (1982) studied genetic and nongenetic variations in temperature tolerance of *E. affinis* measured at three temperatures 10°C, 15°C and 23°C. They showed that physiological variation was similar between males and females. However the experimental protocols used in these earlier studies were not standardized and thus cannot be directly used in comparative studies or used to build individual-based models (Devreker et al., 2007; 2009).

The objective of this paper is to overcome the previously mentioned experimental limitations in using zooplankton as a biological model for ecological and evolutionary studies. According to the importance of cross-generation effects on life cycle traits, we performed in this research a new experimental protocol to study through multiple generations the effects of temperature on the life-cycle plasticity of *E. affinis*. Because the few evolutionary studies focusing on cross generational effects of temperature are based on terrestrial biological models (see Steigenga and Fischer, 2007), we present here the first attempt with an aquatic organism. To take into account the high genetic divergence within the *E. affinis* species complex, three different populations from Europe (2 estuaries) and North America (salt marshes of St Lawrence estuary) were used to capture some of this genetic diversity. In this paper, we tested the effect of cold (7°C) and warm (20°C) temperatures on the life cycle plasticity and the population regulation mechanisms over several generations. We also tested the effects of temperature increase (24°C) on the life cycle of these populations by identifying the similarities and differences in the responses of each selected population.

Materials and methods

Origin of the populations

Three populations of *Eurytemora affinis* belonging to two genetically and morphologically distinct clades (Lee 1999; Lee and Frost, 2002) are used in this study. One population from the North American clade (Lee 1999) was collected in a Salt Marsh (Isle Verte: 48°01’N

69°21'W) of the St Lawrence estuary (Quebec, Canada) in May 2001 and maintained in culture until the start of the experiment. The two other test populations belong to the European clade. They were collected in the low salinity zone of Gironde and Seine estuaries in France. The population from the Gironde estuary was collected in March 2006 near Pauillac (46°00'N- 2°00'W) and the population of the Seine estuary was collected in November 2006 under the Tancarville Bridge (49°26'N-00°16'W). All copepod populations were maintained in the laboratory under controlled conditions according to the protocol detailed in Souissi et al. (2010). (Chapter IV, part 1).

Experimental protocol

In order to study the effects of temperature on the life cycle traits of the three selected populations of *E. affinis* a batch culture protocol was developed. After a previous acclimation during several generations in the mass culture, each batch culture was reared in 2 litre beakers in sea filtered water adjusted to the experimental salinity and maintained in climate chambers at a range temperature of 7°C, 20°C and 24°C. The cultures were fed twice a day by using *Rhodomonas marina* as a unique source of food. At the low temperature of 7°C, the experiment was carried out during 6 successive generations (~6 weeks from eggs to adults' generation time). At 20°C the experiment was conducted during 16 successive generations before increasing the temperature to 24°C (Figure 1).

Here we analyze the last 6 generations conducted at 20°C and the following 5 generations observed after increasing the temperature to 24°C (Figure 1). Each new generation n was started by isolating 40 ovigerous females from the previous generation (n-1). After nauplii were observed, hatching females were removed and the cohort developed until adulthood. The remaining individuals of the generation (n-1) were fixed using buffered formalin for further measurements and enumeration. All experiments were realized in filtered and autoclaved sea water adjusted to the salinity 15, considered to be optimal for *E. affinis* development (Devreker et al., 2004; 2007; 2009).

The following life cycle traits were measured for each generation n from each population k:

1) The density $N_{n,k}$ (total number of individuals produced by the generation)

2) The mortality rate (percentage of dead individuals after one generation compared to a theoretical production of 40 ovigerous females)

3) The sex-ratio (♀/♂ including Copepodid 5 and adult stages)

4) The clutch size (Eggs/sac/♀).

Data analysis

To quantify the effects of the origin of copepod populations and temperature on the different life cycle traits measured, an unbalanced two-way Analysis of Variance (ANOVA) was conducted. When the ANOVA test was significant at the probability threshold level $p< 0.05$, the MULTCOMPARE function available with Matlab Software was used to perform a multiple comparison of means. In addition, for each population, two-sample T-test with pooled or unpooled variance estimates was performed to test the null hypothesis that corresponds to the equality in the means of each life cycle trait between two experimental conditions. Moreover, according to the low number of observations (generations) for each experimental condition (n=5 or 6), intra-population comparisons of the median of all life cycle traits was also realized using the non parametric Wilcoxon rank sum test. All statistical tests were performed using Matlab Software vers 7 (The Mathworks, Inc. 2005).

Results

Multigenerational changes in life cycle traits under temperature effect in three populations of E. affinis

At 7°C the density of the population of *E. affinis* from St Lawrence was always higher than Gironde population and also the Seine population except in F5 (Figure 2A). The Seine population showed the lowest densities from F2 to F4 but rose to highest density in F5 (almost doubeled) and dropped slightly in F6 remaining higher than the first generations (Fig. 2A).

The fluctuation of the densities in the Seine population is always opposite to that of both the Gironde and St Lawrence populations.

At 20°C, from F10 to F14 we observed two different patterns of inter-generation density fluctuations. The Gironde population showed a regular oscillation every generation, whereas the densities in both Seine and St Lawrence populations fluctuated every two generations. In

F15, corresponding to the low food quality episode (LFQ episode), the three populations responded differently (Fig. 2B). The density of Gironde population decreased but in the same sense as the pattern observed in F10-F14. On the other hand, the Seine and St Lawrence populations showed opposite responses breaking the previous pattern of variability. In fact, the density of the Seine population increased whereas that of the St Lawrence population dropped to a critical level showing the lowest density recorded at this temperature (53 individuals). In the following generation (F16) we observed a clear maternal effect response with an attempt of the different populations to recover their regular density. The highest response was recorded in the St Lawrence population.

The increase of temperature to 24°C (+4°C) in all populations induced a perturbation in the density with inter-population differences. On the one hand, Gironde and St Lawrence populations showed an increase in their densities. On the other hand the density of the Seine population decreased. In the first generation at 24°C, the density of Gironde and St Lawrence populations severely dropped, especially for the later population that approached extinction with only a few individuals produced. In contrast, the Seine population density increased slightly in this generation. In the subsequent 4 generations at 24°C, Gironde and St Lawrence densities were mainly stable around 300 individuals whereas the Seine density registered a slight increase (Fig. 2B). But globally in all populations the temperature increase to 24°C (excepting the first transitional generation) engendered lower densities than those observed at 20°C and 7°C.

At 7°C the mortality rate in all populations was low and stable around the mean value of 0.008 d^{-1} (Fig. 2C). At 20°C, the pattern of mortality rate in the three populations showed a decreasing trend in the first five generations (F10-F14, Fig. 2D) which was highly significant for the Gironde population ($R^2=0.94$, $p<0.01$). In the two following generations F15 and F16 associated to low food quality episode (LFQ episode), the mortality rate remained stable except in F15 for St Lawrence population (Fig. 2D).

The increase of temperature to 24°C was accompanied with an order of magnitude increase of mortality rate in the Seine population reaching 0.04 d^{-1}, whereas in the two other populations the mortality rate remained below 0.009 d^{-1}. But, in the subsequent generations, the trends in mortality rate were diverse between populations. The Seine and Gironde populations exhibited opposed tendancies, increasing ($R^2=0.71$, $p<0.05$) and decreasing ($R^2=0.80$,

$p<0.05$), respectively. The mortality rate in the St Lawrence population fluctuated between generations (Fig. 2D).

At 7°C, the sex-ratio in Gironde and St Lawrence populations was often in favor of females except in F4 which was at equilibrium in Gironde population and a bit lower than 1 (0.81) in St Lawrence population. In contrast, the sex-ratio in the Seine population was always male biased (Fig. 2E). At 20°C, the sex-ratio in Gironde population was generally male biased (F10-F14) but reversed since F15 (LFQ episode). The Seine population at 20°C was globally male biased like at 7°C (Fig. 2F). The sex-ratio in St Lawrence population fluctuated approaching the equilibrium in the 5 first generations at 20°C, but since F15 it falls over in favor of females while increasing in the following generations. The increase by 4°C in the temperature returned the sex-ratio of all the populations around the equilibrium in F17. In the subsequent generations at 24°C, the sex-ratio in the Gironde population was always female biased with a highly significant quadratic trend ($R^2=0.9865$, $p<0.01$) whereas the Seine population's sex-ratio dropped in favor of males (mean value was 0.67) after a single generation (F17) female biased. The sex-ratio in St Lawrence population was fluctuating but still mainly in favor of females (except in F19) (Fig. 2E).

At 7°C, the clutch size showed a negative trend through generations for all populations but this was only statistically significant for the Seine ($R^2=0.94$, $p=0.0061$) (Fig. 2G). At 20°C the clutch size of all populations showed clear inter-generation and inter-population variability (Fig. 2H) with general patterns similar to those of the density (Fig. 2B). At 24°C the Gironde population showed the only observed significant increase in clutch size that increased from 19.8 eggs.female^{-1} in F17 to 40.1 eggs.female^{-1} in F21. The St Lawrence population seemed to respond as the Gironde population by reaching a higher clutch size in the last generation (36.43 eggs.female^{-1} in F21) but suffered during the second generation at 24°C (F18) where the density was dramatically low (Fig. 2B). The clutch size in the Seine population at 24°C showed the lowest variability (Fig. 2H).

The effects of all treatments on the density

Figure 3A shows that the mean number of individuals produced by generation of all populations decreased when temperature increased. The two-way ANOVA results confirmed the highly significant effect of temperature ($p<0.0001$) and also a significant interaction between temperature and population origin ($p<0.05$) but no significant differences between populations (Table 1). The MULTCOMPARE test revealed that only the density at the highest temperature (24°C) was statistically different from that at other temperatures when all populations were combined. However, the intra-population analysis revealed that the three temperatures did not affect significantly the Seine population density whereas for Gironde and St Lawrence populations the densities at 24°C were statistically lower than at 20°C and 7°C (T-test, $p<0.0001$ between 24°C and 7°C; $p<0.01$ between 24°C and 20°C).

Globally, mortality increased with temperature increase for all populations except for the Seine population which exhibit a decrease of mortality rate at 24°C (Fig. 3B). Temperature was the only highly significant factor affecting the mortality rate (Anova test, $p<0.0001$, Table 1). The lowest mortality rate was recorded at 7°C and was significantly different from the mortalities at 20°C and 24°C (MULTCOMPARE test). The differences in mortality rates between 20°C and 24°C were only significant for Gironde population (T-test, $p<0.01$). Compared to Gironde and St Lawrence populations, the Seine population did not show any significant difference in mortality rate between all treatments (T-test, $p>0.01$).

The inter-population differences in the sex-ratio (Fig. 3C) was confirmed by ANOVA test ($p<0.01$, Table 1). In fact, for all temperatures, the proportion of males was higher than the females in the Seine population (Fig. 3C). Whereas, for the Gironde and St Lawrence populations, the sex-ratio was in favor of females excepting at 20°C for Gironde population. The MUTICOMPARE test separated the Seine population (the lowest sex-ratio) from the other populations. The interaction between population and temperature was significant (ANOVA test, $p<0.05$). Each population responded differently to each tested temperature. In fact, in the St Lawrence population the sex-ratio did not show any significant difference between the three temperatures, while in the Seine population the sex-ratio was significantly different in the highest temperature compared to the other treatments (T-test: $p<0.001$ between 24°C and 7°C and $p<0.01$ between 24°C and 20°C). For the Gironde population, the significant differences were between 20°C and both 7°C and 24°C ($p<0.05$) where the sex-ratio skewed in favour of males.

Variability of clutch size and its relationship to body length

The mean clutch size decreased with temperature increase without any population effect (ANOVA test $p<0.01$ for temperature, table 1, Fig.3D). Globally for all combined populations the mean clutch size was significantly different between the two extreme temperatures 7°C and 24°C (MULTICOMPARE test). The t-test comparison of the mean clutch sizes for each population revealed only two significant differences ($p<0.05$) for the

Seine (between 7°C and 24°C) and for St Lawrence (between 20°C and 24°C) populations (Fig. 3D).

Figure 4 summarizes the relationship between mean clutch size and mean prosome length for three different populations of *E. affinis* at three temperatures. The mean clutch size was positively correlated with prosome length ($R^2=0.704$, $p<0.01$). This positive trend was opposed to the temperature gradient (Fig. 4).

Effect of density dependence regulatory mechanisms

To test if there is any density-dependence effect on the prosome length, the combined multigenerational data of all populations were plotted in Figure 5. The scatter plot distinguished three different groups associated with the positive gradient of experimental temperatures (Fig. 5). A negative density-dependence effect on the prosome length was only significant at 20°C for all populations ($R^2=0.83$, $p<0.0001$) and for each one of them ($p<0.05$ for the Seine and $p<0.01$ for Gironde and St Lawrence) whereas at the other two temperatures, no clear relationships were noticed (even after log transformation).

Discussion

Context of this study

The effect of climate change on biota is of key concern because the responses of populations and communities are unpredictable. Among key external factors, temperature receives by far the most attention in theoretical and empirical studies. The current debate deals with the capacity of populations to either adapt to temperature increase or to migrate and establish in more favorable habitats (Pörtner and Knust 2007). Although the high number of emerging theories reporting a combination of physiological and demographic studies, the diversity of animal responses (particularly small sized invertebrates) makes difficult the extrapolation of these findings across taxonomic levels and ecosystems.. It is widely admitted that warm adapted organisms (stenotherms) are more competitive than temperate eurytherms (generalist) at higher temperature (Pörtner and Farell, 2008). However the most documented illustrations of the interaction between warming and aquatic ecosystems are based on a few examples of organisms that reached their upper thermal limit (Pörtner and Knust 2007) or living in specific habitats (i.e., polar systems). Recently, Pörtner and Peck (2010) reviewed the possible link (cause and effect) between physiological thermal tolerance of fishes and their consequences at the population and ecosystem levels. In order to confirm or enrich this emerging ecological framework, an increase of the number of biological models, including temperate eurytherm organisms, is needed. Moreover, the experimental procedure should be adequate to permit the synthesis of the empirical observations towards a global and more comprehensive model of the effect of climate change on ecosystems.

Because of the inherent plasticity of most organisms, the consideration of the trans-generational effects is important. So the design of multigenerational experimental protocols was recommended for ecological and evolutionary studies (Klok et al., 2009). The consideration of high number of generations was particularly developed in fast growing bacterium (Bennett and Lenski 2007) and the classical terrestrial models, such as insects (Fischer et al., 2006). In aquatic ecosystems, and particularly for zooplankton, the experiments dealing with more than 2 generations are still rare. We can mention the common garden experiments study dealing with the effect of temperature on different populations of *Daphnia* (Mitchell and Lampert 2000) and the salinity tolerance of two populations of the invading copepod *Eurytemora affinis* (Lee et al., 2007).

When studying the copepod plasticity, it is important to consider at least two generations taking into account the maternal effect observed in other studies for the effects of diatoms on copepod reproduction (Ianora et al., 2004). Holste and Peck (2006) studied the effects of temperature and salinity on egg production and hatching success of the copepod *Acartia tonsa* from the Baltic Sea. They used a thermal gradient table to generate several temperatures. Each population was acclimated to the desired temperature at a rate of 0.6°C day^{-1} (Holste and Peck, 2006). The authors used two trials to cover seven different temperatures. This protocol is accurate but it is only based on the success of the procedure of acclimating copepods. The authors assumed that the acclimation to the extreme temperatures was homogeneous. Other recent studies based on different biological models, confirmed that the question of acclimation needs to be studied carefully and interpreted in the framework of emerging theories of evolutionary thermal biology (Angilletta et al., 2006). The protocols using more than one generation in copepod studies are very rare. Recently Kurihara and Ishimatsu (2008) studied the effects of high CO2 seawater on the copepod *Acartia tsuensis* through three successive generations. The necessity to develop standardized experimental protocols using at least two generations was also suggested for ecotoxicological studies using the copepod *Tigriopus japonicus* (Lee et al., 2008). For *Eurytemora affinis*, Lee et al. (2007)

studied, during 6 successive generations, the physiological reaction norms of the survival at salinity 5 and they showed that a selection of copepods occurred.

In this study, we considered the copepod *Eurytemora affinis* as a biological model to study its life cycle responses to temperature increase. This species has a large geographical repartition and lives in different aquatic habitats in the northern hemisphere. It is mainly encountered in low salinity zone of large estuaries, but can also be present in salt marshes as well as great lakes (Lee et al. 2007). All these aquatic ecosystems and particularly the estuarine habitats of *E. affinis* are very sensitive to hydro-climatic variability (Kimmel et al., 2006). *E. affinis* can be present along the year in the majority of these habitats but decreases during summer where another group of copepods of genus *Acartia*, more thermophile, occur (David et al., 2007a). But we still ignore the exact (or possible) responses of these populations of *E. affinis* to a climate warming. *E. affinis* is a complex species composed by a high number of genetically divergent clades and it’s necessarily to take into account this genetic heterogeneity.

An early study by Heinle (1969) showed the high thermal tolerance of *E. affinis* from the Chesapeake Bay which can reproduce at 30°C. This earlier study showed the necessity to maintain mass cultures of copepods in order to perform accurate experiments on the effects of temperature on the life cycle traits of *E. affinis*. Katona (1970) compared the growth rates of two populations of *E. affinis* from a fresh-brackish pond near Woods Hole (Massachusetts, U.S.A.) and from the Hamble River at Southampton (England, Europe). This study showed some differences of the growth of these two populations at different temperatures, but it is difficult to attribute these differences to their geographical origin (i.e. genetic differences according to Lee, 1999). However, these studies realized more than 40 years ago underlined the scientific concern of comparing *E. affinis* and other congeneric population *E. herdmani* regarding their reactions to high range of experimental temperatures. On the other hand, Bradley and Ketzner (1982) focused on the sources (genetic or non-genetic) of variability in temperature tolerance of the copepod *E. affinis*. They showed that genetic variation was maintained and expressed in both sexes in the cycling environments between 10°C and 23°C, but declined in males in the 23°C constant environment. These experiments suggested that females of *E. affinis* have a greater capacity of internal adjustment to temperature change compared to males.

The estuarine populations of *E. affinis* used in this study are selected from two well studied estuaries in France: Gironde and Seine. These populations encountered different local conditions leading to several selection pressures and most probably different life cycle strategies. The maximum of abundance of *E. affinis* occurs in early spring in Gironde (David et al. 2007b) whereas the population of the Seine is more dominant along the year and showed a maximum of density in late spring early summer (Mouny and Dauvin 2002; Devreker et al. 2008). The most important difference is the reproductive strategy of *E. affinis* showing a very low fecundity in the Gironde (David et al. 2007b) compared to the Seine population with much higher fecundity (Chapter II, part 2). Although these two populations belong to the European Clade (Lee 1999) a more detailed analysis at the European scale showed significant genetic differences between these populations (Winkler et al., in revision). Thes differences in habitats, trophic conditions and also the seasonality could be amplified by the genetic differences between these populations. In order to compare the possible genetic effect, another transatlantic population originated from the St Lawrence Salt Marshes (belonging to the Atlantic clade according Lee (1999)) was used. This population offered a third replicate (under the hypothesis of the absence of genetic effect on the life cycle traits) as well as control population living since several generations in the laboratory. The processes of selection could occur in this population giving another interesting context to compare 'laboratory induced' selection under low fluctuating optimal conditions compared to the mechanisms of selections that may occur in both estuaries Gironde and Seine.

In a scenario of climate change, two critical phases in the development of *E. affinis* have to be considered. *E. affinis* is a cold temperate species showing a clear decrease in its abundance during summer. We can thus focus on the effect of temperature increase during the summer season. But the annual cycle of the species should be considered because the responses of the different individuals could vary during the year (i.e., a strong stress during winter could compromise the strength of the annual peak (Devreker et al. 2010). For this reason two critical temperatures were considered, a summer like temperature corresponding to the upper thermal limit (20°C) and a winter like temperature (7°C) corresponding to the pre-blooming phase (before spring-early summer maximum). The climate change scenario was only applied to the upper thermal limit (+4°C) because increasing the lower temperature will only accelerate the development of *E. affinis*. In fact, Devreker et al. (2007, 2009) showed that the development of *E. affinis* at temperatures 10°C and 15°C was optimal and only the effect of high salinity was recorded. The simulated summer temperature 24°C was not observed in the natural habitat of *E. affinis* in the Seine Estuary but can be observed in Gironde (David et al. 2007b) during the minimum abundance of *E. affinis* and also in the Salt Marshes (Winkler, unpub data). We consider that this scenario is very realistic regarding the climate change projections (IPCC 2007).

The life cycle strategy of E. affinis at low and high temperatures

All three populations of *E. affinis* used in this experiment were capable to maintain an average high density (equivalent to 400-500 individuals.L^{-1}) compared to field concentrations. The mortality rate was very low (~0.008 d^{-1}). At low temperature, females reached the largest sizes following the temperature size rule and produced the greatest clutch size (~40 eggs.$female^{-1}$). The decrease in clutch size across generations (all populations combined, R^2=0.60; p<0.001), accompanied by a significant decrease in mortality rate (R^2=0.39, p<0.05), contributed to stable population densities. It is possible that the decrease in individual size is associated with the high density of the populations rather to any possible inbreeding depression in culture.

At 7°C as well as at 20°C, *E. affinis* from all populations showed a high fitness and was capable of maintaining high density during almost all generations by using different regulatory mechanisms. At 20°C, a clear density dependence regulatory mechanism of growth and fecundity acting through clutch size (R^2=0.294, p<0.05) was observed. The regulatory mechanisms at 20°C could be explained by the high number of generations used to acclimatize the 3 populations at 20°C. In fact, when temperature increased to 24°C following a moderate warming scenario, we noticed some differences between populations. The more stable population was that of the Gironde. The St Lawrence laboratory culture suffered in the first generation after temperature increase and approached extinction. The Seine population, in F17, showed a direct response to the thermal stress (+4°C). The following generation (F18) expressed the maternal effect response where we noticed a drop. F17 density combined the previous maternal effect and integrated the rapid change of the environment (temperature).

Inter-population differences

Even if the mass culture techniques and the experimental protocols realized in the earlier studies were not standardized, they showed that the highest temperature treatments increased the proportion of females (Bradley and Ketzner 1982; Katona 1970). Our results showed that the sex-ratio in the European populations of *E. affinis* increased in favor of females at the highest temperature of 24°C. Moreover, the high salinity seems to increase the mortality of males. The determinism of sex-ratio may be related to the genotype, the environment or the interaction between both factors, but the distinction between all these categories is not always clear (Jimenez-Melero et al., 2007). Devreker et al. (2007) studied the individual development of *E. affinis* at 10°C and 15°C and different salinities. They observed a male-biased sex-ratio which could be associated to additional stress engendered by individual rearing conditions. The individual-based protocols offer valuable information, particularly to quantify all reproductive processes of egg-bearing copepods, such as *E. affinis* (Devreker et al. 2008). But for studying cross generation effects it is necessary to develop another experimental protocol. We proposed a general multigenerational protocol that can be applied to any other copepod species. We designed the protocol to test simultaneously basic theories of thermal adaptation and life cycle ecology. We favored batch culture rather than the individual protocol which we used in previous studies (Devreker et al. 2007, 2009). In order to avoid the allee effects (negative fitness due to low population size) the initial number of ovigerous females was relatively high (40) for the experimental volume (2 l). A very low number of females will increase the stochastic noise in the life cycle patterns (Souissi et al., 2004). We tested experimentally in the laboratory different initial densities of ovigerous females to initiate a batch culture and found that the number of 40 is a good compromise to study the life cycle traits through different generations.

This standardization of the experimental protocol confirmed the high performances of *E. affinis* populations whatever their origins, because, for example, the low fecundities recorded in Gironde estuary were overcome in the laboratory when food was available. This suggests that *E. affinis* in the Gironde is limited by local unfavorable conditions. Consequently using only field data compiled from different studies will not be adequate to compare the life-cycles of different populations of *E. affinis*. In spite of this standardization, some differences between populations were identified. The most significant one corresponded to the sex-ratio. The seine population showed a male biased sex-ratio in the field (Devreker et al. 2008, 2010) as well as in the laboratory cultures using either individual (Devreker et al., 2007) or batch cultures (this study, other observations). It seems that the male biased sex-ratio has a genetic origin in the Seine estuary. Understanding the reasons for such strategy will be interesting. For the other life cycle traits, we showed that the population from the St Lawrence salt marshes approached two times the extinction. This means that this population may have become sensitive and may have experienced some selection in the laboratory as this population is maintained in the laboratory since 2001. We could not identify the direction of this selection because it occurred in a mass culture, but we suggest that the population maintained at relatively stable temperature (10-15°C) suffered more from temperature increase. It is surprising that this pattern occurred only after several generations at 20°C. It is probable that the low food quality event played a role in increasing the fluctuations of *E. affinis* from the St Lawrence. We show that the response of the Seine population to the low food-quality episode was different and opposite to that observed in other populations (mother and grandmother effect responses). Moreover, the intersexual individuals provoked by this event were only observed for the Seine population and at the low temperature (Souissi et al., 2010). This suggests that the determinism of sexes in the Seine population could be different from the other tested populations.

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Tables

Table 1. Two-way ANOVA results for 4 measured life cycle traits: Density (total number of individual produced after one batch per generation), mortality rate (proportion of died individuals per day), sex-ratio (female:male) and clutch size (eggs.female^{-1}). The tested factors are the origin of the population (Seine, Gironde and St Lawrence) and temperature (7°C, 20°C and 24°C).

Trait	Factor	d.f.	MS	F	P level<
Density	1-population	2	6808.71	0.164	0.8497
	2-temperature	2	956696.27	22.986	**<0.0001******
	1 x 2	4	127599.28	3.066	**0.0285***
	Error	36	41621.29		
Mortality	1-population	2	0.00007	0.922	0.4068
	2-temperature	2	0.0015	19.248	**<0.0001******
	1 x 2	4	0.00015	1.841	0.1424
	Error	36	0.00008		
Sex-ratio	1-population	2	0.560	7.936	**0.0014****
	2-temperature	2	0.070	0.984	0.3837
	1 x 2	4	0.199	2.812	**0.0396***
	Error	36	0.071		
Clutch size	1-population	2	6.114	0.050	0.9506
	2-temperature	2	675.439	5.606	**0.0076****
	1 x 2	4	61.111	0.507	0.7306
	Error	36	120.488		

Figures

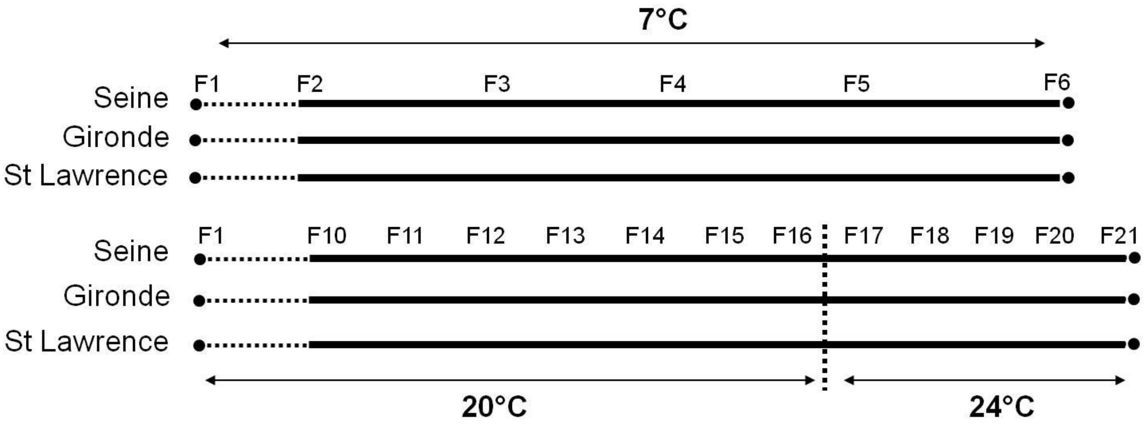

Figure 1. Experimental design for the multi-generation protocol used for three different temperatures 7°C, 20°C and 24°C and three geographically and genetically divergent populations of *E. affinis* isolated from the Seine estuary (France), Gironde estuary (France) and salt marshes of St Lawrence estuary (Canada).

The lines with labels present the number of generations followed for each studied population of *E. affinis*. The starting dashed part designate the acclimation period of the different copepod cultures. The thick parts indicate the generations used in this study for each population. The thin double arrowed lines show the temperatures used in each multi-generation experiment and the vertical dashed line specify the transit to 24°C.

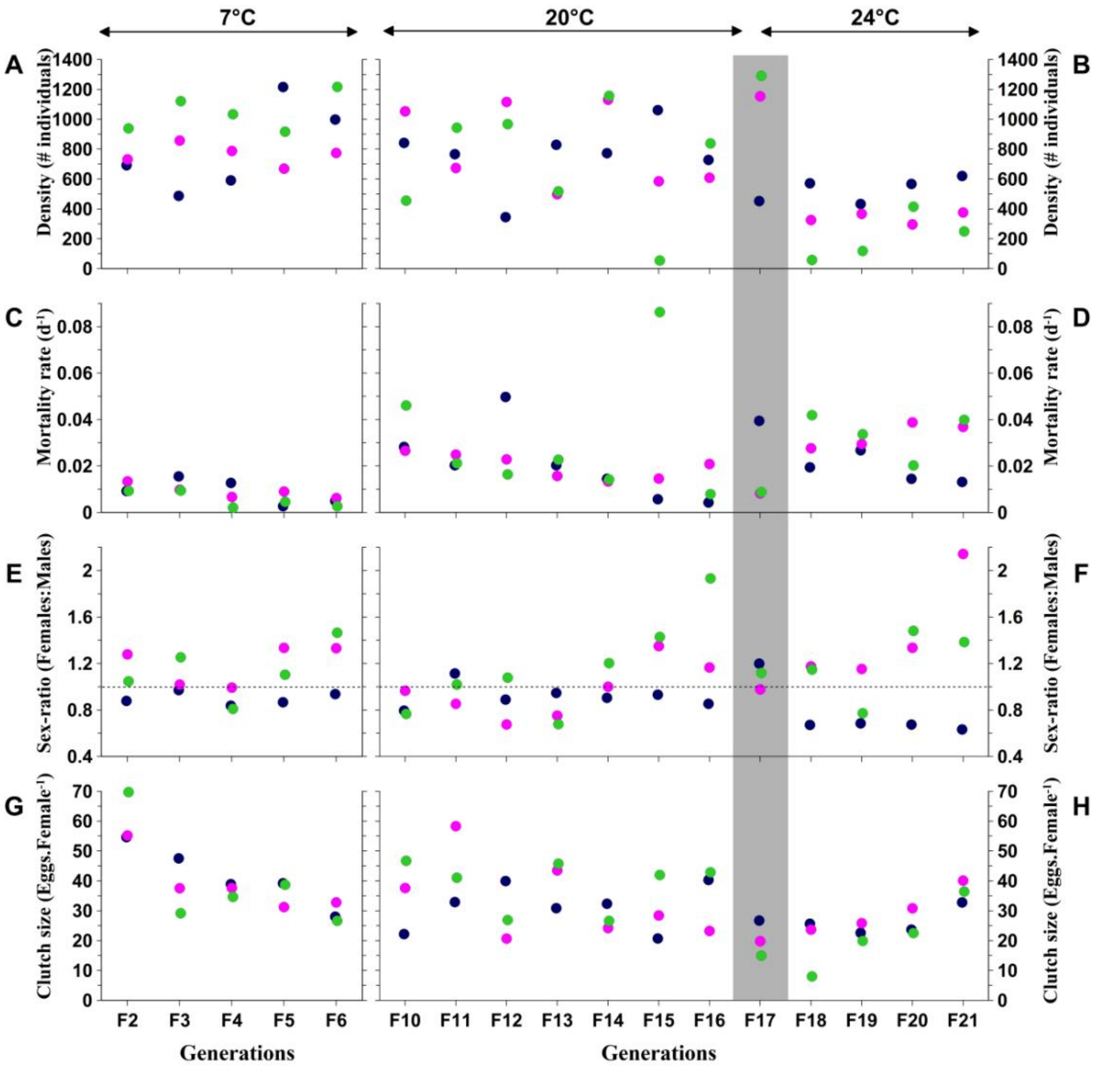

Figure 2. Multigenerational changes in life cycle traits under temperature effect in three populations of *E. affinis*: **A, B**: the density variability in *E. affinis* populations as a function of the different generation at 7°C (A) and at 20 °C then 24°C (B). **C, D**: variability of the mortality rate in *E. affinis* populations through multiple generations under temperatures 7°C (C), 20°C and 24°C (D). **E, F:** variability of the sex ratio in *E. affinis* populations through multiple generations under temperatures 7°C (C), 20°C and 24°C (D). The continuous dashed line designates the 1:1 sex ratio. **G, H:** the clutch size variability in *E. affinis* populations as a function of the different generation at 7°C (G) and at 20 °C then 24°C (H). The thin double arrowed lines at the top of the graphs shows the different temperatures studied. The grey vertical band specifies the first generation after the transit of the copepod population cultures to 24°C. The blue circles indicate the data of the Seine population, the pink circles show the result of the Gironde population and the green circles designate the data of the St Lawrence population.

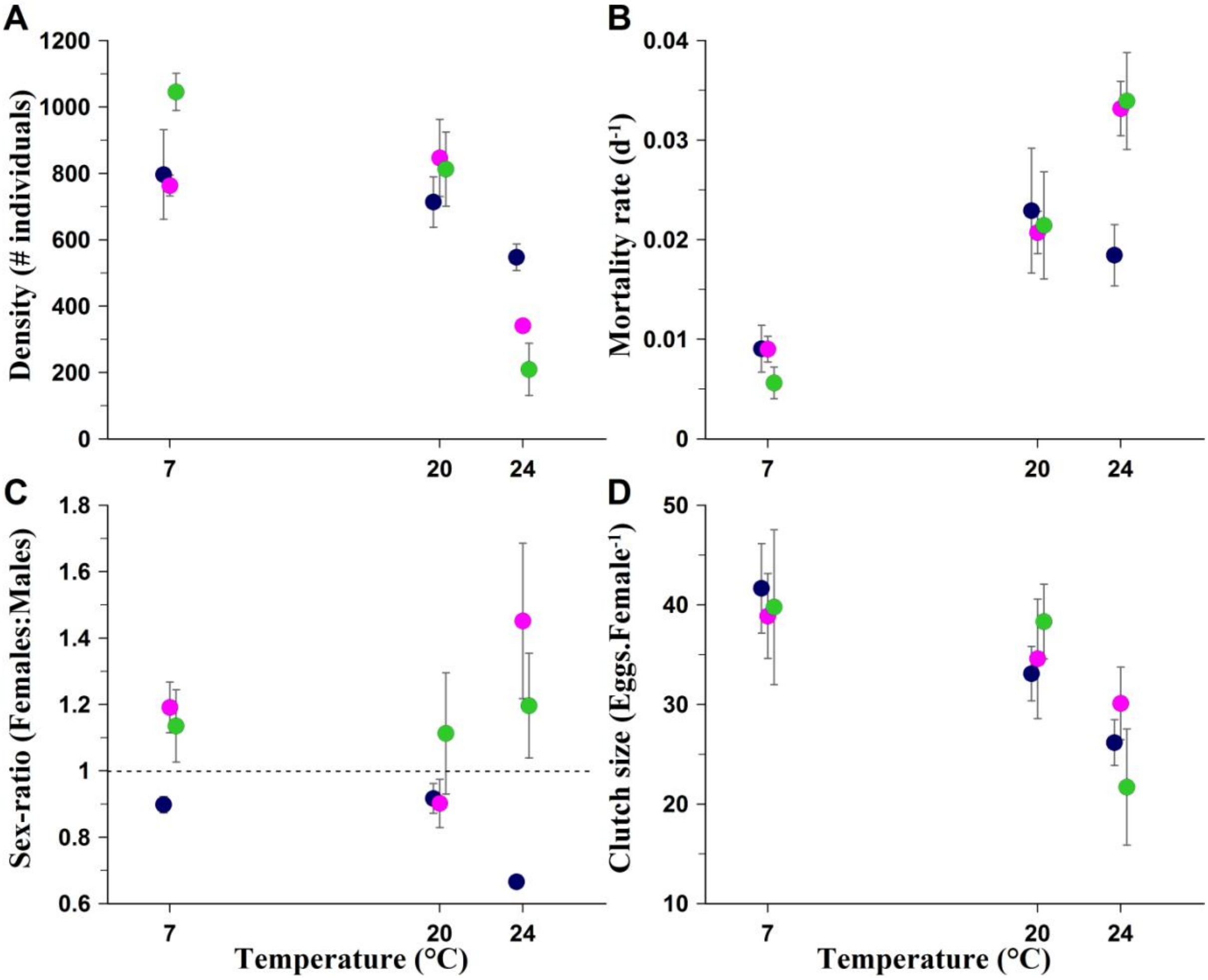

Figure 3. Mean data of some life cycle traits (density (A), mortality rate (B), sex ratio (C) and clutch size (D)) of the different studied populations as a function of the temperature.

The colors of the circle labels designate each population as in figure 2. Vertical bars show the standard error.

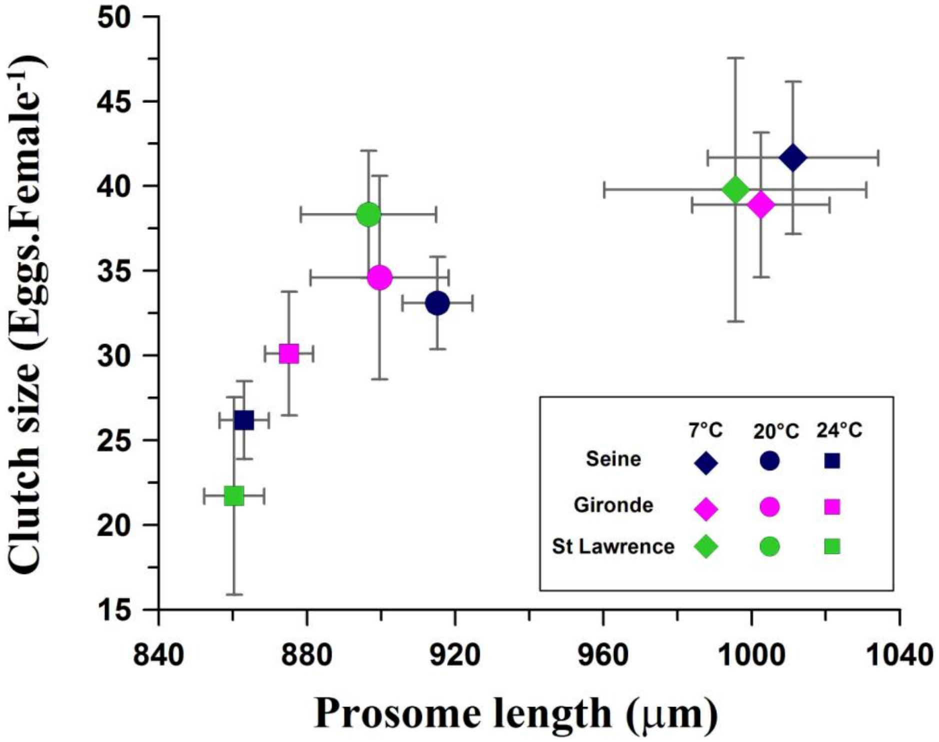

Figure 4. Relationship between the means female's prosome length and clutch size at the different temperatures. The labels designate the different temperatures and the color indicate the population origin as indicated in the rectangle. Error bars present the standard error.

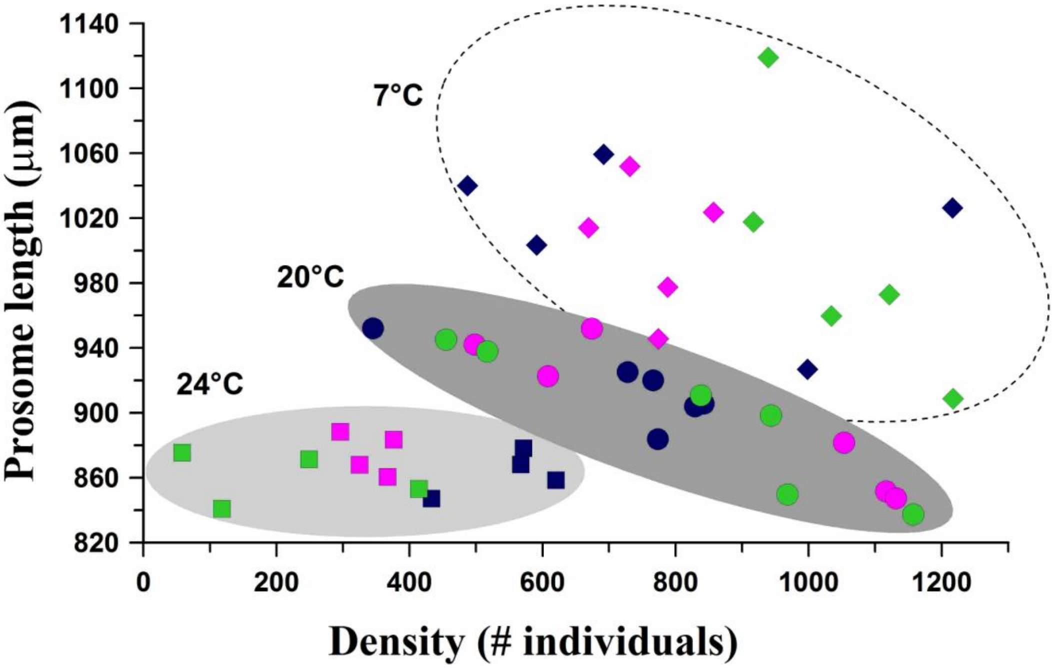

Figure 5. Density-dependence effect on the prosome length of *E. affinis* at different thermal conditions. The data represent the average values in each generation. The labels shape and color are as in figure 4. The ellipsoids delimitate the different temperature groups. The dashed line ellipsoid designate the group of data of 7°C, the grey ellipsoid indicate the group of data of 20°C and the light grey ellipsoid contains the group of data of 24°C.

Chapter II: Reproductive strategy of the different populations of *Eurytemora affinis*

Part 1: ***Experimental test of the reproductive trade-offs of Eurytemora affinis (Copepoda: Calanoida)***

Introduction

The reproductive strategy is one of the most important outcomes of evolutionary history as it serves to maximize parental fitness (Stearns 1992). Reproduction requires resources that can be limited by several external and/or internal factors and it is these factors that determine the total reproductive effort. Reproductive effort can be best described as the total number of offspring and the reproductive investment in each. Females invest a quantity of resources in their offspring (i.e., reproductive effort), but are faced with a trade-off: should the female produce a larger number of smaller offspring or a smaller number of larger offspring? Recognition of trade-offs between these two fitness components has been well documented in terrestrial ecology (Price 1974, Roff 1992, Carrière 2001). Most of these studies reference the model proposed by Smith and Fretwell (1974) based on two intuitive assumptions: 1) the existence of a trade-off between offspring number and size, i.e. when energy invested in individual offspring increases (resulting in larger offspring) the total number of offspring decreases; and 2) a positive relationship between individual offspring size and its fitness. This model has proven more applicable to species that produce numerous small offspring without parental care, thus we can apply this model to a group that exhibits these traits: crustacean zooplankton species.

Approximately 1400 references cite the Smith and Fretwell (1974) model; however, only few examples dealt with zooplankton in general with a small number of papers concerning copepods in particular. The trade-off between egg size and reproductive output (fecundity or reproductive effort) was analysed for a combination of copepod groups consisting of free living and parasitic taxa (Poulin 1995, Caley et al. 2001). The only study dealing with a single species was performed by Timi et al. (2005) showing no evidence of trade-off between fecundity and egg volume in a parasitic copepod. The absence of trade-off in this parasitic copepod could be due to the high resource availability that allowed good allocation to both egg size and number (Timi et al. 2005). Most free living copepods face resource limitations, thus, a trade-off between egg size and number is expected. Empirical observations showed that the carbon content of individual egg scaled with female carbon content (Kiørboe and Sabatini, 1994). A further studied showed that egg carbon content could be empirically modelled as a general power law (i.e., linear in log-log plot) of egg diameter among several species of copepods (Uye and Sano, 1995). The consideration of carbon content is common in copepod ecology because one of the key reproductive parameters is the specific egg production rate calculated as the proportion of female body mass (usually expressed in terms of carbon content) invested in the clutch mass (in term of total eggs carbon) per day. The trade-off between egg and clutch size in copepods has received less attention. From the few examples available, the data indicate that concentration affects the trade-off between offspring number and egg size in the marine copepod *Euterpina acutifrons* (Guisande et al., 1996). However, this study did not test the Smith and Fretwell (1974) model directly, only confirming that egg size can decrease with egg number until a certain threshold value, a vale corresponding to a minimal egg size. Measured egg size in copepods also shows seasonal variability. For example, Liang and Uye (1997) showed a clear seasonal pattern in egg diameter of the egg bearing copepod *Pseudodiaptomus marinus* with larger eggs produced by bigger females during which time of year?. Generally, copepods are fast growing organisms and their reproductive output is often considered as an estimator of the quality of their environment. But no deep experimental study was devoted to test the ecological and evolutionary significances of the trade-off occurrence between clutch and egg sizes.

From ecological point of view, copepods living in highly variable environments and exhibiting a clear seasonality in their life history patterns (i.e., high reproduction during spring in temperate environments) should display a particular life history strategy with regard to offspring size and number. The investment of more energy into individual offspring would lead to improved fitness of the offspring when the optimal seasonal temporal window occurs. Jamieson and Santer (2003) showed that large eggs of the freshwater copepod *Cyclops kolensis* delivered during the first clutch contributed disproportionately to the population because they produced fast growing nauplii and large copepodites. Therefore, examination of the reproductive strategy of females with regard to offspring size and number improve our understanding of the life cycle strategies of copepods and lead to more realistic individual based models to be used for…. ????.

The examples mentioned above suggest that egg size among egg bearing copepods shows variability over the course of a season. The egg-bearing copepods employ a reproductive strategy that results in much lower egg mortality because eggs are carried in an egg sac (Hirst and Kiørboe, 2002). In addition, egg bearing copepods offer an advantage to test the trade-off theory because ovigerous females can have their size (maternal control) and reproductive effort (including clutch and egg sizes) measured.

The egg-bearing copepod we will use in this study is the estuarine copepod *E. affinis*, the dominate zooplankton species in several estuaries and other aquatic ecosystems in the northern Hemisphere (Winkler et al 2008, Andersen and Nielson 1997). In macrotidal estuaries, *E. affinis* evolved adaptations to keep the bulk of its population in the low salinity zone and is exposed to high variability of both salinity (at tidal scale) and temperature (at all scales). Several field and laboratory studies showed that the clutch size of *E. affinis* is highly variable and could be affected by food, temperature and salinity (Ban 1994; Devreker et al. 2009). But no experimental study, either in the field or in the laboratory, examined the partitioning of the reproductive effort in terms of clutch size and egg size.

We use a standardized experimental protocol to follow the development of a laboratory reared? *E. affinis* population during several generations under various temperature and salinity conditions (see Chapter I for more details). We used a population originally collected from the Seine estuary (Devereker et al. 2009) and made the hypothesis that the reproductive strategy of this population should exhibit a trade-off between clutch and egg size under the differing conditions.

We are intended to answer the following questions:

i) What are the effects of temperature and salinity on the total reproductive effort of *E. affinis*?
ii) Is there any trade-off between clutch size and egg size in a single population of *E. affinis*?
iii) What are the effects of acclimation and warming on the reproductive strategy of *E. affinis*?

Material and Method

Experimental conditions

The population of the copepod *E. affinis* collected from the Seine estuary (France). All individuals used in the different experiments were isolated from the field in November 2006 in the low salinity zone under the Tancarville bridge (49°26’N-00°16’W). The copepods were then split into two separate, laboratory populations, one that was acclimated to a lower temperature (7°C) and another to a higher temperature (20°C) over several generations. At 20°C, two salinities were used, one optimal (15) and another stressful (25) for the reproduction of *E. affinis* (Devreker et al., 2009). In these experiments, the multi-generation protocol was used (Souissi et al. 2010). The details of this protocol and the follow up of the different generations were detailed in Souissi et al. (2010). The details of this protocol and the follow up of the different generations were detailed in Souissi et al. (2010) (Chapter IV, part1) and in the chapter I. In this study, we consider each generation as a replicate of the experimental conditions. In order to increase the environmental gradient and include additional possibilities of temperature and salinity combinations, all initial conditions (T7S15, T20S15 and T20S25) were transferred to a higher temperature of 24°C and followed during 5 subsequent generations.

Excepting a single generation (F3 after the transfer of T7S15 to T24) where the number of ovigerous females was very low and consequently they were used in totality to initiate F4 in T24(7)S15 all other generations produced enough ovigerous females to make the required measurements. Between 20 to 40 ovigerous females were sorted in each generation to measure all morphological and reproductive traits. Every female sorted was observed under an inverted microscope then the prosome length and width were measured as detailed in Souissi et al. (2010). Afterward, the clutch size was counted by tearing the sac carefully and individual measurements of 5 to 15 eggs per sac were done to determine egg size. All the size measurements were conducted using ImageJ software.

Selection of the reproductive traits

The fecundity (F) of *E. affinis* was estimated by the clutch size (number of eggs.female^{-1}) whereas two variables were used to estimate egg size: egg diameter (ED) and egg volume (EV). Egg volume was calculated based on a spherical model. The total reproductive effort (RE) was then defined as the total volume of the clutch (clutch size x mean EV). Because reproductive traits in copepods are usually correlated to female body size, the prosome length (PL) and the prosome volume (PV) were used as measures of female size.

Statistical analyses

We used the Pearsons's correlation coefficient to test the possible relationships between all variables measured using original data and also log-transformed. All correlations with log-transformed variables gave lower R^2 than the original data; therefore only linear relationships were selected. Because the reproductive output of copepods is often determined by female body size, we first verified this statement. The correlations between reproductive traits and female body size were very similar, when either PL or PV was used. As a consequence, only relationships with female PL, which is the most used variable to describe body size in copepods, were retained.

Because simple linear regressions between clutch size, egg size and total reproductive effort (dependent variables) and female PL (independent variable) gave the most significant relationships (compared to multiple linear regressions), we decided to perform a simple residual analysis to test the existence of trade-offs between egg size and number. A particular interest was given to the relative relationship between ED and clutch size (CS), after removing the effects of female body size.

All statistical analyses were done with Matlab software.

Results

The largest mean PL was obtained at the lowest temperature and PL decreased as temperature and salinity increased (Table 1). The CS followed the same pattern in almost all conditions except T24(7)S15, which had the lowest measured CS. The ED was highest at 7°C and showed no discernable difference at 20°C regardless of salinity (Table 1). After the transfer to 24°C, the ED decreased in the T24(7)S15 treatment; increased for the T24S15 treatment and remained stable at the T24S25 treatment. For all treatments, the ED was highest from copepods that originated from the 7°C treatment (Table 1).

Female PL explained 85.1%, 70.0% and 25.6% of the total variance in RE, CS and ED, respectively (Table 2). The relatively low r^2 obtained between ED and PL is due to the high dispersion of the data observed in all generations (Fig.1B). ED showed no significant relationship with CS (r^2=0.032, p=0.362). CS (Fig.1A) and RE (Fig. 1C) showed a significant positive trend with PL.

We regressed the residuals of the linear regressions of both CS and ED versus PL and the result was a negative relationship (Fig. 2). This linear regression explained almost 27% of the total variance in CS using ED as a predictor (Table 2). This negative relationship between residuals suggests that for a given PL, females with small CS (i.e., symbols below the regression line in Fig. 1A) tend to produce larger eggs (i.e., symbols above the regression line in Fig. 1B). The reverse is also suggested, where females with larger CS tend to produce smaller eggs (Fig. 1A and 1B). The data from the populations initially acclimated to the low temperature (7°C) were situated in the same area of the graph that corresponded to smaller CS and larger ED. In contrast, populations reared at 20°C (both salinities) were situated in an opposite area of the graph, an area that corresponded to larger CS and smaller ED. After transfer to 24°C, the ED of these populations did not appear to change (i.e., residuals of ED vs PL were close to 0) compared to other temperatures, did show high variability in CS (CS remained smaller than other treatments). We separated the initial and final experimental conditions and performed the same regression between the residuals of CS and ED. The residual analysis was highly significant for the initial conditions (r^2=0.638, n=14, p<0.001); however, the relationship was not significant after the experiment was run (r^2=0.002, n=14, p=0.875). This suggests that the negative slope observed in Fig. 2 was driven primarily by the initial conditions, which suggest and trade-off. However, when conditions become stressful, no trade-off was observed (Table 2).

Discussion

Previous studies on the reproduction of the copepod *E. affinis* often focused on the fecundity or carbon content of the produced clutches. These studied converted CS into carbon using a single estimation of the carbon content of an individual egg of constant size (Heinle and Flemer, 1975). For example, Hirche (1992) compared the reproductive strategies of *E. affinis* and to the broadcast spawner *Acartia tonsa* using a fixed ED for both species (82 µm and 73 µm, respectively). This approach is obviously flawed in light of the observed variability in egg size that reflects a possible change in the reproductive investment of the female. For example, Crawford and Daborn (1986) showed that the egg diameter of *E. herdmani* (congeneric species of *E. affinis*) living in a turbid estuary varied between 82 and 99 µm. Despite only 4 measurements of ED (see Table 1 in Crawford and Daborn 1986), a significant negative correlation was obtained with the CS (R=0.94, n=4, p<0.05) suggesting a possible existence of a trade-off between these two reproductive traits. Therefore, it is possible that *E. affinis* populations exhibit such a trade-off and our results strongly support this hypothesis.

Our study applied a multi-generational approach to determine if a trade-off between clutch size and egg diameter existed for *E. affinis*. This protocol has been used successfully to address other hypotheses concerning *E. affinis* ecology, such as acclimation to environmental conditions and life history traits (Chapter I). The experiments conducted in this study were performed on a single population of *E. affinis* that originated from the Seine estuary and aiming to test a global warming scenario that can lead to an increase in both temperature and salinity (IPCC 2007).

The measure of clutch size, commonly used in the literature (Ban 1994, Uye & sano 1995, Uye & Sano 1997), was linearly related to female PL (Figure 1A). In contrast to Ban (1994) who found a power relationship between PL and CS in *E. affinis*, our fit was linear. These differences could be due to experimental differences between the two studies. Ban (1994) focused on the effect of food concentration on reproductive output for a single isolate of copepods, while our protocol was based on a batch culture and multigenerational observations. In both cases, a general positive trend between CS and PL was observed demonstrating higher fecundity in larger females. When the total reproductive effort was calculated (the product of CS and mean EV (i.e., CV)), its relationship with female PL was also positive (see Fig. 1C and Table 1) as a direct consequence to the EV changes. In fact, if the EV was held constant, the relationship between either CS or CV (CS x EV) versus PL results in the same regression coefficients and slopes?

We found that the mean egg diameter of *E. affinis* can vary from 91.96 µm to 104.19 µm, an overall increase of 45.1 % in EV. The variability of ED was mainly explained by female size and less so by temperature and salinity (see Fig. 1B). The positive relationship between PL and ED was present in all combined and separated conditions (see Table 2). This result suggests that *E. affinis* can adjust both the size and the number of its offspring. In order to remove the effect of female size on reproductive traits, we used the residuals from regression with PL and the presence of a trade-off between egg size and egg number existed. In fact, the significant negative trend observed between residuals (see Fig. 2 and Table 2) confirmed that the allocation of the reproductive energy in egg number and egg size was not done independently. The negative trend of Fig. 2 means that *E. affinis* females investing more energy in their offspring (i.e., at low temperature 7°C) produced relatively smaller clutches whereas ovigerous females with relatively bigger CS (i.e., at 20°C) invested less in their individual offspring which resulted in smaller eggs. To our knowledge, this study provides the first experimental confirmation of the presence of a trade-off in the reproductive strategy of an egg-bearing copepod.

The phenomenon of a trade-off between egg number and egg size has been observed in terrestrial invertebrates such as butterflies (Fisher et al. 2006) and crickets (Carrière and Roff, 1995). Only one detailed study at the species level exists for copepods and this species is parasitic (Timi et al. 2005), thus our ability to complete a comparative analysis for pelagic copepods is limited. At higher taxonomic levels, Caley et al. (2001) suggested that total reproductive effort (RE) and egg size in copepods did not evolve independently. The high heterogeneity in the literature values and the high variability in the residuals (see Caley et al. 2001 Fig. 1) resulted in a low amount of variability of egg size (<11%) explained by RE. Caley et al. (2001) recognized the limitations of using an estimation of the total reproductive effort that was not independent from the estimation of the egg size and suggested that future studies should focus on the simultaneous allocation decisions rather than sequential ones as well as other genetic or selective mechanisms. We confirmed their conclusions at the specific level by using *E. affinis* as a biological model. The observed trade-off between egg size and egg number was driven by the initial conditions individuals experience during the first several generations in culture. It is possible that this finding resulted from a selection process where individuals experiencing the same conditions during multiple generations produce the same trade-off. Moreover our experimental protocol was designed to test a possible effect of a climatic scenario (i.e., increase in temperature and/or salinity). We showed that after temperature increased to 24°C (due to a possible warming or to an artificial heat shock) the trade-off between egg size and egg number observed in the initial conditions vanished. This means that either the high temperature of 24°C disrupted the reproductive trade-off because it was very stressful or that the population requires a much longer acclimation period to adapt to this new environment. Whatever the exact mechanisms causing to the absence of a reproductive trade-off at very high temperature, the future studies on the possible effects of warming (or climate change in general) on the life cycle traits should consider the inter-dependence of reproductive traits.

The experimental temperatures used in the initial conditions (7°C and 20°C) correspond to the observed extremes in the low salinity zone of the Seine estuary during the seasonal growth of the population of *E. affinis* between late winter and early summer. Consequently, *E. affinis* should optimize its reproductive strategy at both temperatures to ensure the optimal development of its offspring. At the lower temperature (7°C), *E. affinis* females grew slowly but showed the lowest mortalities among larger sized individuals (Chapter I, part 2) . The positive relationship between female size and fecundity has been shown for many ectotherms (References?), including *E. affinis* (Chapter I), Our study confirmed that fecundity was enhanced with larger female size *and* the quality of individual offspring is also enhanced. The upper thermal limit (20°C) corresponds to the decline of the *E.* affinis population in the Seine estuary (Mouny and Dauvin 2002). At this temperature in the lab experiments, the trade-off between egg size and number favored the production of more smaller-sized eggs. In the Seine estuary, the *E. affinis* population faces its highest mortalities due to summer high temperatures and increased predation (Chapter I, part 2, Mouny et al. 1998). A short-term shift in reproductive strategy could improve the number of individuals that survive in this unfavorable season. Moreover, we showed that *E. affinis* development occurs at 20°C in the laboratory when all other external factors were removed. Even when temperature was raisedo 24°C (including the artificial heat shock treatment from 7°C to 24°C) all populations were able to survive and reproduce without showing any apparent trade-off pattern. A detailed examination of the data (Fig. 2) confirmed that the population originated from 7°C kept the same initial trade-off after transfer to 24°C. This suggests that the initial acclimation to 7°C selected a strategy that favored female investment in the individual offspring, even after 5 generations. On the other hand, the 4°C increase in the other treatments acclimated to the upper thermal limit (20°C) seemed to disturb the trade-off strategy during the five subsequent generations. In global warming scenario, the summer population of *E. affinis* may face such an increase in temperature and their response to this challenge may be impacted significantly according to our results.

In conclusion, we confirmed in this study that *E. affinis* ovigerous females appear capable of adjusting their egg size in addition to their clutch size. The variability of these two reproductive traits was highly correlated to female body size. However, when the effect of body size was removed, we found that females appeared to display a clear trade-off between size and number of offspring, a trend that also depended upon the environmental conditions. This study is the first experimental test of Smith and Fretwell’s (1974) model to an egg-bearing copepod and our data supports this model for the copepod *E. affinis*. As in the case of Caley et al. (2001), we demonstrated that the allocation of reproductive energy was not simply sequential, rather….. The extrapolation of our results in an ecological context appear to be related to the observed seasonality of the *E. affinis* population in the Seine estuary. *E. affinis* is a cryptic species composed by different populations living in fragmented habitats in the northern hemisphere (Lee 1999). Therefore the consideration of its inter-population variability should lead to a better understanding of the role of their reproductive strategies.

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Tables

Table 1. Experimental conditions of *E affinis* reared in the laboratory with the number of generations (# Gen.) and the total number of ovigerous females (# Ind.) observed at each experimental condition. Inter-generations means (±SD) of female prosome length (PL), clutch size (CS) and egg diameter (ED) are shown. T.= Temperature, S.= Salinity.

	T - S	# Gen. (# Ind.)	PL (µm)	CS (eggs. ♀$^{-1}$)	ED (µm)
Initial conditions	T7S15	4 (121)	999.14±50.54	38.42±8.02	100.12±1.41
	T20S15	8 (205)	899.82±44.77	32.84±8.03	93.59±0.61
	T20S25	7 (182)	875.38±47.14	30.05±8.85	93.31±1.41
Final conditions	T24(7)S15	5 (112)	867.78±36.49	21.75±4.06	98.95±3.66
	T24S15	5 (196)	860.33±12.98	26.29±3.98	95.05±1.70
	T24S25	5 (135)	847.48±9.99	22.04±4.89	93.64±1.26

Table 2. Results of the linear regression analysis (Y=aX+b) between reproductive traits of *E. affinis* as dependent variables (CS, ED and CV) and PL (independent variable) as well as the relationship between the residuals from these regressions (CS vs PL) and (ES vs PL).

CS: Clutch size, ED: Egg diameter, CV: Clutch volume, PL: Prosome length, ES: Egg size, Resid. Residual.

	Y=aX+b		**a (slope)**	**b (intercept)**	**n**	**R^2**	**Adj-R^2**	**p**
All conditions	Y=CS	X=PL	0.118 ± 0.031	-76.806 ± 27.835	28	0.700	0.689	<0.0001
	Y=ED	X=PL	0.027 ± 0.019	71.548 ± 16.483	28	0.256	0.228	<0.01
	Y=CV	X=PL	$6.7\ 10^{-5}$ ± $1.14\ 10^{-5}$	-0.047 ± 0.010	28	0.851	0.845	<0.0001
	Y=Resid. (CS vs PL)	X=Resid. (ES vs PL)	-0.874 ± 0.582	0.014 ± 1.551	28	0.268	0.240	<0.01
Initial conditions	Y=CS	X=PL	0.105 ± 0.040	-62.889 ± 36.696	14	0.733	0.710	<0.0001
	Y=ED	X=PL	0.034 ± 0.021	63.733 ± 19.510	14	0.511	0.470	<0.01
	Y=CV	X=PL	$6.5\ 10^{-5}$ ± $1.08\ 10^{-5}$	-0.045 ± 0.010	14	0.935	0.930	<0.0001
	Y=Resid. (CS vs PL)	X=Resid. (ES vs PL)	-1.502 ± 0.712	-0.041 ± 1.590	14	0.638	0.607	<0.001
Final conditions	Y=CS	X=PL	0.068 ± 0.126	-34.599 ± 108.290	14	0.102	0.027	0.265
	Y=ED	X=PL	0.091 ± 0.069	17.190 ± 59.521	14	0.408	0.358	0.014
	Y=CV	X=PL	$6.5\ 10^{-5}$ ± $6.05\ 10^{-5}$	-0.045 ± 0.052	14	0.316	0.259	0.037
	Y=Resid. (CS vs PL)	X=Resid. (ES vs PL)	-0.085 ± 1.143	-0.008 ± 2.616	14	0.002	<0.000	0.875

Figures

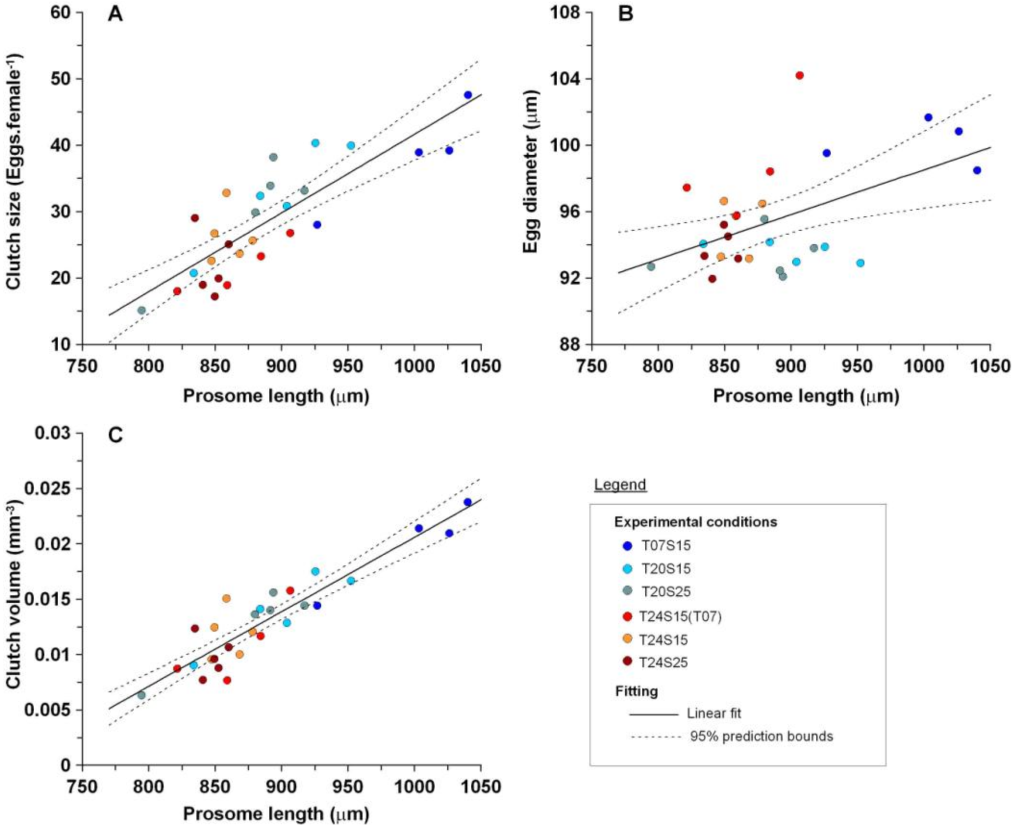

Figure 1. Relationship between *E. affinis* female prosome length with clutch size (A), egg diameter (B) and clutch volume (C). The rectangles contain the labels of each population used from laboratory experiments. The solid line is the regression line and the dashed lines are the 95% confidence interval..

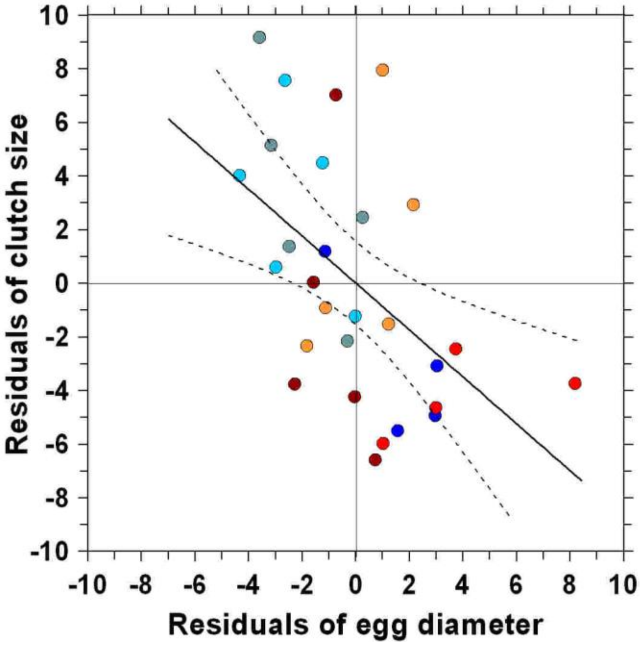

Figure 2. Relationship between residuals of linear regressions of mean egg diameter and clutch size against female prosome length. The solid line is the regression line and the dashed lines are the 95% confidence interval. The grey lines intersect the origin (0,0). The labels of each population are the same as in Fig. 1.

***Part 2*: Do trans-Atlantic populations of Eurytemora affinis have the same reproductive patterns?**

Introduction

The allocation of resources to reproduction is a life-history trait that is subject to competing selection pressures. Reproductive allocation can therefore be modeled by defining the optimal allocation of resources to reproduction in relation to other traits such as survival and somatic growth. The result is often a trade-off resulting in either the production of many, small offspring or a few, larger offspring (Charnov 1989; Roff 1992; Stearns 1992; Caley et al. 2001). Caley et al. (2001) hypothesized that a negative correlation existed between fecundity and offspring size in copepods in contrast to most other studies that have assumed these traits to be independent. Caley et al. (2001) tested this hypothesis by using an artificial selection experiment and field studies on natural selection based on a phylogenetic database involving 105 families of free living and parasitic copepods. One of the potential shortcomings of this effort was that it did not distinguish between models of sequential versus simultaneous allocation of the reproductive effort in Copepoda, despite the existence of these allocation strategies in the Copepoda. Our study of *Eurytemora affinis* was meant to investigate life-history trade-offs in more detail..

We confirmed the existence of a trade-off between egg size and number in the calanoid copepod *Eurytemora affinis* experimentally (Chapter II, part 1). We showed that the reproductive strategy was opposite between cold acclimated population and warm acclimated one. Moreover, we confirmed that the acclimation history to either cold or warm environments differentially affected the trade-off when the initial conditions were modified (i.e. increase of temperature. The experimental study was based on a single population of *E. affinis* from the Seine estuary situated at intermediate latitude in the geographical range of the species. In the present study, we focused on field populations of *E. affinis* from a wider geographical range. The goal was to test for the existence of a trade-off between egg size and number. The study was necessary because *E. affinis* is a cryptic species composed of several populations that mostly evolved independently in fragmented habitats (Lee 1999). For comparison to the Seine population, we considered 5 populations in Europe and 5 populations in North America.

We chose to address the following questions:

i) Are reproductive effort and its two components (clutch size and egg size) linearly related to female body size (as shown experimentally)?

ii) Is-there a trade-off between egg size and egg number in the different populations of *E. affinis*?

iii) Is-there any difference in clutch size and egg size between European and North American populations?

Material and Method

The selected estuaries in Europe and North America are representative of the transatlantic distribution of *E. affinis* as well as the diversity of their estuarine habitats (Fig. 1). These sites offer a latitudinal gradient in similar macrotidal European estuaries and two contrasting latitudes in much larger estuarine ecosystems in North America: the St Lawrence estuary and the Chesapeake Bay (Fig. 1).

Regarding the temporal scale, the combined samples cover all seasons for both continents. However, sampling in the St Lawrence estuary was not possible during winter and early spring and the highest number of samples was taken from the low salinity zone in the Seine estuary (Table 1). In fact, the Seine is considered as a reference estuary because of its intermediate latitude and the existence of several experimental studies on the reproductive strategy of its *E. affinis* population (Chapter II, part 1; Beyrend-Dur et al., 2009; Devreker et al. 2009; Dur et al. 2009).

Measurements of the ovigerous females' body size, clutch size and egg size as well as statistical analyses were described in the part 1 of the Chapter II.

Results

The relationship between female prosome length and the three reproductive traits; clutch size (CS), egg diameter (ED) and clutch volume (CV) was highly significant for the combined data (Table 2). Figure 2 showed a high dispersion in the different scatter plots particularly in the PL-CS plane, whereas the PL-ED plane presented relatively a low dispersion. For the reproductive traits, in all field data, female PL explained 33.4%, 50.3% and 52.4% of the total variance in CS, ED and CV, respectively.

When the residuals of the linear regressions of both CS and ED against PL were plotted, a negative trend was obtained (Fig. 3). In this case, the linear regression explained 26.3% of the total variance and this was statistically significant (Table 2). This means that, for a given PL, *E. affinis* ovigerous females with many eggs (i.e., symbols above the regression line in Fig. 2A) tend to produce relatively smaller eggs (i.e., symbols below the regression line in Fig. 2B) and *vice versa*.

The residual analysis presented in Fig. 3 discriminated between the populations in Europe and in North America. In fact, most of the data from the European populations, particularly the three French estuaries (Seine, Loire and Gironde), were situated in the quarter corresponding to relatively smaller CS (negative ordinate) with larger eggs (positive abscissa). Whereas, the data from North American estuaries showed much higher dispersion (Fig. 3), with an average position situated in the quarter corresponding to ovigerous females producing bigger CS and smaller eggs. The two remaining quarters contain few data from both continents and show residuals with either positive or negative signs. This means that egg size and egg number in these cases varied in the same direction. However, most of these data were positioned close to the origin of the axes (0,0), thus were not different from zero.

This analysis suggested that the reproductive strategy of *E. affinis* populations differed from both continents. Therefore, we split the dataset into two transatlantic groups and analyzed the relationships between the PL and both reproductive traits (CS and ED) and prosome width (PW) within these groups. In both transatlantic groups, the variability in PW was explained (almost 90% of total variance) by the variability in PL (Fig. 4C). For the relationship between PL and both reproductive traits, the data from European estuaries showed stronger correlations than the North American data (Table 3). Based on the regression, the North American ovigerous females were wider, on average, than European ovigerous females (Fig 4C). However, for a given female PL, the CS was higher in North American populations that, overall, produced smaller sized eggs. The linear regressions between ED and PL gave very different slopes (Fig. 4B); in contrast, the other regression lines were parallel (Fig. 4A, C). Both slope and intercept were not statistically different between populations (Table 3). But when the data were represented by the different ratios (PW:PL), (CS:PL) and (CS:ED), clear differences between transatlantic populations were confirmed for the two first ratios (Fig. 5). We noticed that the differences in CS:ED approached significance ($p=0.057$) suggesting that a higher number of samples may confirm these differences.

Discussion

The differences between the number and size of offspring among genetically related organisms could be due to several factors occurring at different temporal scales. In fact, depending on the specific life-history of a species the reproductive responses of individuals to their environmental constraints may be different. One of the most studied reproductive strategies in animals is the optimal offspring size theory (Smith and Fretwell, 1974). This theory suggest that when resources are limited, a trade-off should occur between offspring number and size. Many species do not follow the expected negative relationship between offspring size and number in natural or laboratory conditions (Camargo et al., 2008). Few studies of reproductive trade-off in the Copepoda have specifically addressed optimal size theory. Rather, most empirical studies on copepod reproduction have focused on the effects of different external factors on clutch size or carbon content (Ban 1994, Uye & Sano 1995, Liang and Uye 1997). The relation between female body size and demographic processes including fecundity (Hirst and Kiørboe 2002) has also been conducted, but in most of these studies, the importance of egg size variability has been overlooked. Consequently, these published data were not applicable to optimale size theory models (i.e., Smith and Fretwell 1974) in order to test the division of reproductive allocations between clutch number and egg size. The lone exception was the comparative study done by Caley et al. (2001) which analyzed a set of reproductive trait data from parasitic and free living copepods. They found a negative pattern between reproductive effort and egg size and concluded that a simultaneous allocation decision, rather than a sequential decision should be explored in Copepoda.

We have demonstrated that a negative trade-off between egg size and egg number exists in *E. affinis* after accounting for female body size (Chapter II, part 1). Our data suggested that *E. affinis* is capable of adjusting its reproductive allocation and of partitioning its resources between egg size and egg number. The study further showed the capacity of *E. affinis* to shift reproductive strategy after a certain period of acclimation. A strategy expressed at low temperatures was opposite that of a strategy expressed at high temperatures and we interpreted this as an adaptive response of *E. affinis* to the variable thermal conditions encountered in an estuary.

In the natural environment, *E. affinis* is known to be a cryptic species composed of different isolated populations that could exhibit divergent life-histories that have developed under diverse selection pressures (Lee 1999, 2000; Winkler et al. 2008). It has been demonstrated using phylogenetic analysis that *E. affinis* populations are composed of several distinct clades (Lee 1999). This analysis also suggests that reproductive isolation occurred between these populations for quite some time (Lee et al., 2007). No direct evidence has been shown to test the consequences of this isolation on the reproductive strategy in terms of trade-off between egg size and number. We used 10 populations of *E. affinis* from Europe and North America (see Fig. 1) on the basis of the literature stating their genetic and morphological divergence (Lee and Frost 2002; Winkler et al. 2008) to test for the existence of life-history trade-offs that differ by population. The objective of this study was the assessment of the validity of our experimental results on the occurrence of optimal reproductive allocation in the natural habitats of *E. affinis*.

The mean egg diameter of *E. affinis* from all combined estuaries showed high variability and ranged from a minimal value of 78.37µm (in the Chesapeake Bay) to a maximal value of 99.80 µm (in the Seine estuary). The differences between these extremes in egg size were of 27.35% in ED which corresponded to 106.3% in EV. These differences were much higher than those observed in the laboratory conditions, which were 13.3% and 45.1% for ED and EV, respectively. However when we considered each transatlantic group separately, these disparities decreased. For European populations, the differences in egg size amplitudes were similar to the experimental ones, with 14.08% and 48.48% for ED and CV, respectively. In the North American populations the differences were more important with 21.00% and 76.95% in ED and EV, respectively.

The patterns of reproductive traits (CS, ED and VC) with PL were all similar to our experimental results with a high variability between populations. By using the residuals from regression against PL, it was revealed that the variability between transatlantic populations was mainly due to differences in their reproductive strategies, i.e. differences in egg number and size. The European populations generally invested more resources into the production of larger individual offspring (i.e., large egg size. On the contrary, the strategy of ovigerous females from North American appeared to center on the production of larger clutches of smaller eggs.

The separation between transatlantic populations and the analysis of their respective reproductive patterns regarding female body size, confirmed the differences in their reproductive traits. Camargo et al., (2008) recommended considering historical constraints on reproductive variables in order to analyze phylogenetic distances. We confirmed in this study that a clear shift in the reproductive strategy appears to have occurred between phylogenetically different sub-population of *E. affinis* (Lee 1999). To our knowledge, this is a first evidence of the existence of such a shift in a trade-off between egg size and egg number that might be due to evolutionary processes.

The North American populations of *E. affinis* considered in this study were mostly composed by the freshwater invading Atlantic clade (excepting St Jean Port Joli site that contained the non-invasive North Atlantic clade of *E. affinis*) (Winkler et al. 2008). This group is marked by the ability to adapt to very low salinities. This, combined with a shift in the reproductive strategy may play a central role in the wide distribution of this clade in North America. Recent investigation by Alekseev et al. (2009) confirmed that the same Atlantic clade of *E. affinis* was observed in North-East Baltic Sea. The reproductive strategy of this clade underlined in this study could be an advantage to these populations to extend their geographical distribution.

A North-South gradient of European estuaries corresponded to a clear gradient in the reproductive output (in terms of clutch size) of *E. affinis*; however, the general pattern in reproductive allocation did not show high variability compared to the North American case. The different populations of *E. affinis* sampled in Europe shared similar habitats, found most often in the low salinity zones of macrotidal estuaries. The observed differences among European populations affected principally the reproductive effort (CS? ES?), whereas its allocation as well as female's morphology followed the same pattern. Consequently, the use of *E. affinis* from the Seine estuary as a reference population for other Europe populations seems appropriate (Chapter II, part 1). The North American populations of *E. affinis* showed a high dispersion (see Fig. 2-4) that could be due to the large size of the studied estuaries as well as the different abiotic gradients that populations can encounter.

In addition to the existing genetic separation between these transatlantic populations of *E. affinis*, the clear differences in the general morphological traits and also in the reproductive tactics questioned the status of these populations as? a species complex. We may be witnessing a continuation of a speciation process that started millions of years ago. More detailed morphological observations of the transatlantic populations of *E. affinis* confirmed that morphological differences do exist and suggest speciation has occurred within this cryptic species (Alekseev and Souissi, (2011))

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Tables

Table 1. Sampling sites of ovigerous females of the copepod *E. affinis* in European and North American estuaries. The geographical position (latitude and longitude), the sampling dates and the number of observations in each site were mentioned.

Continent	**Estuary**		**Latitude – Longitude**	**Dates (Nbr of observation)**				
				2006	**2007**	**2008**	**2009**	**Other years**
Europe	**Elbe**		53°32’06N - 09°47’31E	17 Mar (20)				
	Scheldt		51°21’06N - 04°14’58E	4 Apr. (19)	11 Apr. (11)	6 Apr. (11)		
	Seine		49°28’33N - 00°27’54W	23 May (19)	10 Oct (21) - 6 Nov (16)	8 Mar (19) - 10 Apr (27) 8 Jun (34) - 8 Jul (21) 20 Sept (11) - 4 Nov (15)	25 Feb. (20)	10 Feb. 2010 (18)
	Loire		47°17’23N - 02°01’52W	18 Apr. (20)	15 Nov. (31)	16 Apr. (9)	25 Jun (16)	
	Gironde		45°14’80N - 00°43’50W	20 Apr. (20)			15 Apr. (15)	
North America	**St Lawrence**	**Isle Verte**	48°00’20N - 69°25’50W		18 May (15) - 12 Jun (20) 11 Jul (26)		22 May (7)	
		St Jean Port Joli	47°12’59N - 70°16’22W		11 Jul (6)	15 Jul (19)	20 May (20) 2 Jun (33)	
		Montmagny	46°59’26N - 70°33’13W		14 Jun (20)		May (7)	
		Berthier sur Mer	46°56’07N - 70°44’07W		11 Jul (1)	15 Jul (17)	29 May (5)	
	Chesapeake Bay		39°23.81'N - 76°03.32'W			15 Dec (19)	6 Feb (22) - 28 Apr (11)	May 2002 (6) Apr 2003 (4)

Table 2. Results of the linear regression analysis (Y=aX+b) and their statistics applied to the reproductive traits of *E. affinis* as dependent variables (CS, ED and CV) and PL (independent variable) as well as the relationship between the residuals (CS vs PL) and (ES vs PL).

Y=aX+b		a (slope)	b (intercept)	n	R²	Adj-R²	p
Y=CS	X=PL	0.117 ± 0.056	-64.577 ± 49.610	38	0.334	0.315	<0.001
Y=ED	X=PL	0.034 ± 0.011	60.653 ± 10.119	38	0.503	0.489	<0.0001
Y=CV	X=PL	$6.4\ 10^{-5} \pm 2.06\ 10^{-5}$	-0.041 ± 0.018	38	0.524	0.510	<0.0001
Y=Residuals (CS vs PL)	X=Residuals (ES vs PL)	-2.514 ± 1.423	-0.041 ± 4.828	38	0.263	0.243	<0.001

Table 3. Results of the linear regression analysis (Y=aX+b) and their statistics applied to the *E. affinis* in Europe and North American estuaries using as dependent variables (CS, ED and PW) and PL (independent variable).

Y=aX+b		a (slope)	b (intercept)	n	R²	Adj-R²	p
EUROPE							
Y=CS	X=PL	0.172 ± 0.042	-124.421 ± 38.865	21	0.795	0.785	<0.0001
Y=ED	X= PL	0.022 ± 0.012	72.834 ± 10.911	21	0.446	0.417	<0.001
Y=PW	X= PL	0.458 ± 0.072	-96.982 ± 66.397	21	0.904	0.899	<0.0001
NORTH AMERICA							
Y=CS	X= PL	0.171 ± 0.110	-97.774 ± 91.628	17	0.423	0.385	<0.01
Y=ED	X= PL	0.036 ± 0.025	57.339 ± 20.948	17	0.386	0.345	<0.01
Y=PW	X= PL	0.519 ± 0.100	-107.669 ± 83.885	17	0.890	0.883	<0.0001

Figures

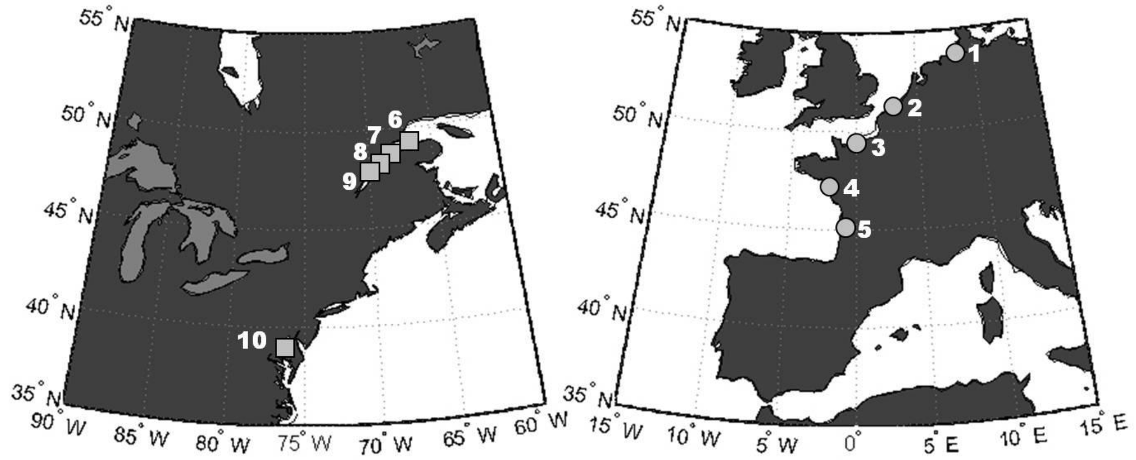

Figure 1. Map of the sampling locations in Europe (1: Elbe, 2: Scheldt, 3: Seine, 4: Loire and 5: Gironde) and North America (St Lawrence estuary sites: 6: Isle Verte, 7: St Jean Port Joli, 8: Montmagny and 9: Berthier sur Mer; and 10: Chesapeake Bay)

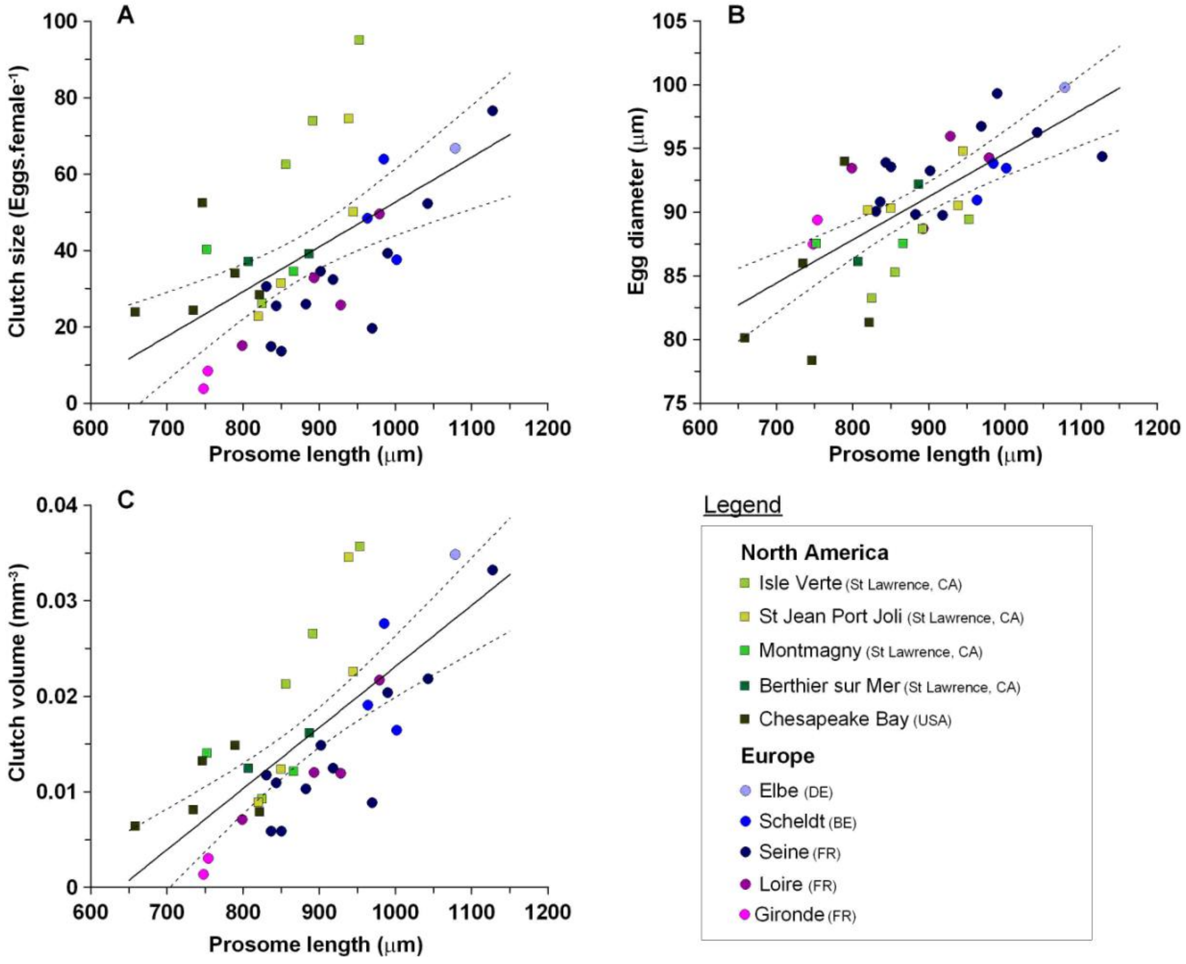

Figure 2. Relationship between *E. affinis* females prosome length with clutch size (A), egg diameter (B) and clutch volume (C). The rectangles contain the labels of each population used from Europe (circles) and Northe America (square). The continuous line is the fitting and the dashed lines are the 95% interval of confidence of the fitting.

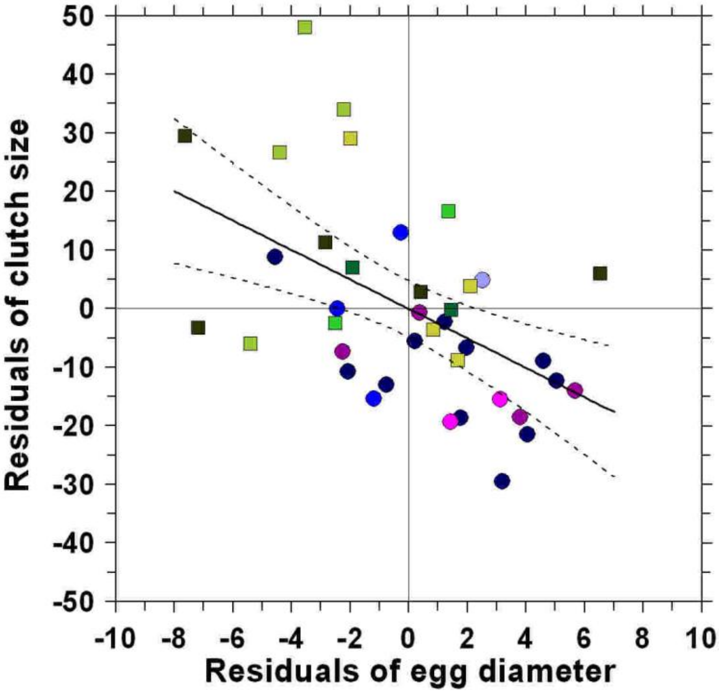

Figure 3. Relationship between residuals of linear regressions against female prosome length of mean egg diameter and clutch size. The continuous line is the fitting and the dashed lines are the 95% interval of confidence of the fitting. The grey lines materialize the centered axes with origin (0,0). The labels of each population are the same as in Fig. 2.

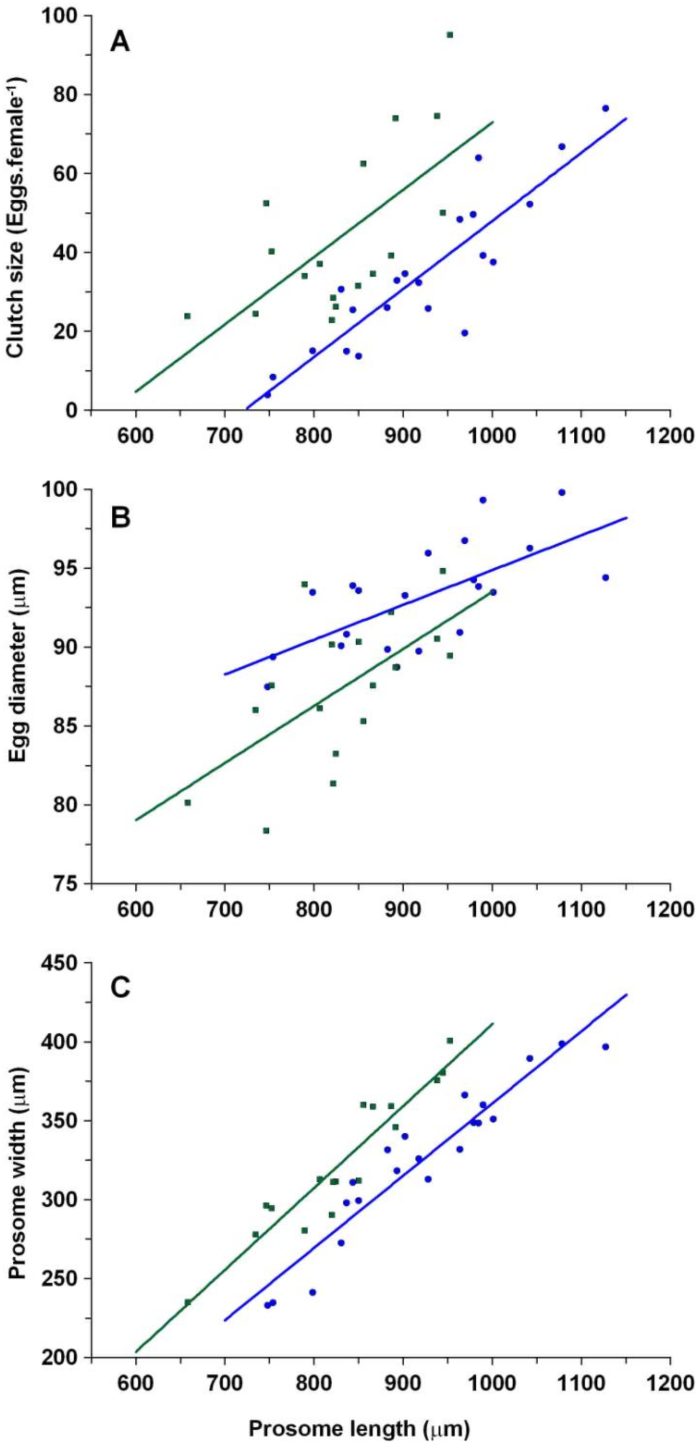

Figure 4. Relationship between *E. affinis* females prosome length with clutch size (A), egg diameter (B) and prosome width (C). The observed data from European populations (blue symbols) and north American population (green symbols) and their linear regressions (continuous lines) are shown. Results of the regression are shown in Table 2.

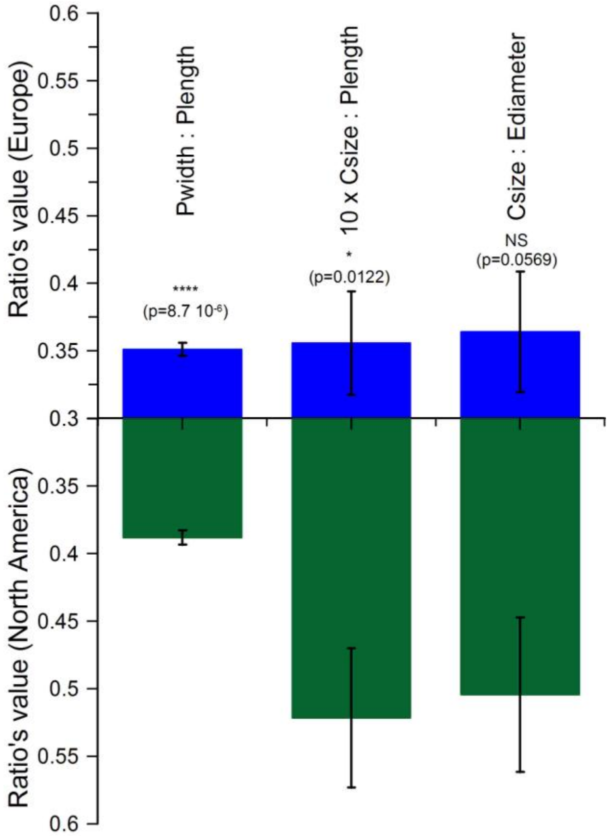

Figure 5. Comparisons between reproductive traits ratios (PW:PL, CS:PL and CS:ED) in European populations (blue bars, above) and north American ones (green bars, below). Vertical bars represent standard errors. The differences between transatlantic populations are realized using t-test.

<u>Part 3</u>: The egg packing constraint in different populations of the calanoid copepod Eurytemora affinis

Introduction

Calanoid copepods are divided in two groups according to their reproductive modes; the broadcast spawners or free spawning copepods and the egg brooding or bearing copepods. In the last group, the female copepod retains her eggs until hatching in a sac attached to the first genital segment of her urosome (Kosobokova et al 2007). Most of the studies dealt with the clutch size and the egg size without direct attention to the egg sac itself and the relationship between its morphology and the reproductive strategy of the female.

The egg sac has a very important role in the life history of egg bearing copepod since it contributes to the survival of the species by insuring the egg carrying (Logerwell and Ohman 1999) but it contributes also to the mortality of the species by increasing the predation risk. In fact, ovigerous females are more exposed to visual predators (Bollens and Frost 1991) and also have less escape ability leading to the increase of predation risk (Svensson 1992, Logerwell and Ohman 1999). Logerwell and Ohman (1999) showed also the importance of the egg sac position and the interaction with the swimming behavior which influence the predation risk more than the body size.

Some authors doubted the presence of true egg sac with external membrane in calanoid copepods and stated that it is just an egg mass formed by secretions that paste the eggs together and attach the mass to the genital somite (Huys and Boxashall 1991, Mauchline 1998). On the contrary, other authors showed that there is a real egg sac in calanoid copepod with clear evidence of the presence of an external membrane (Hopkins 1977, Ohman and Townsend 1998, Kosobokova et al 2007). The presence of a membrane induces the fact of packing the eggs in the sac. As in many taxa which female retain their eggs in a body cavity (e.g., turtle), the egg bearing copepods with true egg sac face the constraint of eggs packing. The packing constraint can affect the egg size, the clutch size and the reproductive trade-off (Congdon and Gibbons 1987, Glazier 2000, Beck and Beck 2005). Beck and Beck (2005) proposed the first study of the packing constraint effect on the reproductive allocation in relation to the egg size for turtles and copepods, and found that the nature of this relationship depends on the presence of packing constraint. For copepods, Beck and Beck (2005) analyzed different taxonomic groups including divergent branches such as parasitic and free living copepods, by using composite phylogenies. To our knowledge, such analysis of packing constraints has not yet been studied at lower taxonomic levels like order or genus.

We hypothesize that the packing constraints should concern primarily egg-bearing copepods because broadcast spawners do not carry their eggs.

Among the brooding species, the calanoid copepod *Eurytemra affinis* offers a good model to test the presence of egg packing constraints in ovigerous females and its ecological significance in their habitats. This egg bearing copepod is a dominant species in estuaries and salt marshes of the Northern hemisphere from coastal region of North America to Europe and Asia (Lee 1999). Lee and Frost (2002) stated that *E affinis* is a species complex composed of distinct populations. In fact, this species has divergent clades between North America and Europe at the gene level (Lee 2000; Winkler et al., 2008; Winkler et al., in revision) and at the physiological response level (Lee 1999, Beyrend-Dur et al 2009). This divergence among the species complex is due to an evolutionary separation inducing an adaptation to their highly fluctuating habitats (Winkler et al., in revision).

In this study, we aimed to understand the mechanism of egg production in relation to the strategy of the female in her habitat and tried to answer these questions:

1) What is the link between the egg sac and the genital urosomal segment?

2) What is the relationship between the egg sac size and its packing state and the reproductive allocation of the female?

3) Is there an effect of temperature or salinity on egg packing constraints?

4) Is there an inter-population and/or inter-clade differences in eggs packing constraints?

Materials and methods

Samples of *E. affinis* were collected from different estuaries in Europe and North America (Table1). These samples are representative of the northern transatlantic distribution of the species in estuaries (Fig 1, Table1).

The highest number of samples comes from the Seine estuary which we consider in this study as a reference site for *E. affinis* population because of its situation at intermediate latitude in Europe (Fig 1). In addition to the *in situ* samples collection, cultures of *E. affinis* population originated from the Seine was also conducted at different conditions of temperature and salinity as detailed in the part 1 of the chapter I. In fact, three sets of experiments were conducted over several generations at different conditions with one copepod culture at 7°C and another at 20°C. Both cultures were set at salinity 15. The third culture was performed at salinity 25 and at 20°C. For all these cultures of *E affinis*, the temperature was increased to 24°C and the experiments followed through five more generations (see Souissi et al., 2010; Chapter I, part 1; Table 2). From each generation of all culture conditions, 20 to 40 ovigerous females were taken (Table 2). Similarly, from each *in situ* sample, 10 to 30 ovigerous females were sorted depending on their availability in the samples.

Whatever originated from the laboratory or from the field, every female sorted was observed under an inverted microscope then all traits of interest for this study were measured. Indeed, the prosome length and width were measured as detailed in Souissi et al. (2010) and the genital first segment of the urosome width was also considered. A special care was attributed to the egg sac; a great attention was given to the choice of a complete egg sac with all the eggs inside the sac surrounded with the membrane (Fig 2). Then the maximum length was measured from the attachment part in the genital segment to the end of the sac (the limit of the membrane when it is visible) (Fig 2). The width measurement was considered in the middle of the sac from one side limit of the membrane to the other side. Afterward, the clutch size was counted by tearing the sac carefully and 5 to 15 eggs per sac were measured (see Chapter II, part 1). All the measurements were realized using ImageJ software.

Volumes estimation

Both Prosome and Egg sac shapes were assimilated to an ellipsoid; consequently, their volumes were calculated by using the following equation (ellipsoid volume formula):

$$Xv = \frac{4}{3}\, pi.\left(\frac{Xl}{2}\right).\left(\frac{Xw}{2}\right)^2$$

Where, Xv is Prosome (or Egg sac) volume (mm^3), Xl is Prosome (or Egg sac) length (mm) and Xw is Prosome (or Egg sac) width (mm).

The clutch volume (mm^3) of each female was estimated by multiplying the total number of eggs (clutch size (CS)) by the mean egg volume (Ev) considered as spherical of each female clutch

$$Cv = CS\ .\ Ev$$

Packing constraint estimation

To quantify the packing constraint, we suggest a simple index that we call “Packing Pressure Index” (PP index) by computing the difference between the observed values of the egg sac volume (*ESv*) and the clutch volume (*Cv*):

$$PP\ index = ESv - Cv$$

When PP index is negative, we consider that it is clear indication of strong packing constraint. However, in order to take into account the presence of the free volume between eggs in their distribution in the sac (knowing that. all eggs without heavy packing constraints are spherical), we used two reference lines: $y=x$ and $y=1.5x$. When observed data are below the line $y=x$ (i.e., $ESv \leq Cv$), we consider that there is a strong packing constraint (SPC). When observed data are between the two lines (i.e., $Cv < ESv \leq 1.5Cv$), we consider that there is an intermediate packing constraint (IPC) between clutch volume and sac volume and when observed data are above the $y=1.5x$ line (i.e., $ESv > 1.5Cv$), we consider that there is no any packing constraint (NPC).

Statistical analysis

Statistical analysis for the correlation between all traits compared and the fitting were realized using the Matlab software.

Results

Relationship between the reproductive effort and morphological reproductive traits

The clutch volume was significantly correlated to the prosome volume in both cases; *in situ* (Fig 3A) and laboratory (Fig 3B). The dispersion of the data plotted in the prosome volume – clutch volume plane around the linear regression lines were very low in a single population under 6 experimental conditions (R^2=0.874, p<0.0001) and high (R^2=0.620, p<0.0001), when all estuaries were combined (Fig. 3A).

As expected, the egg sac volume showed a strong positive relationship with the clutch volume, in all *E. affinis* populations from field (Fig.3C, R^2=0.817, p<0.0001) and laboratory (Fig 3D; R^2=0.929, p<0.0001). For *in situ* data, we noticed that the European populations were closer to the regression line than the North American ones, whereas, in the laboratory conditions, almost all generations'data were situated within the confidence limits of the regression lines (Fig. 3D).

Because egg sac volume was a good proxy of clutch volume (Fig.3C,D), we logically obtained a good relationship with the prosome volume (Fig. 3E,F) but with more dispersion for field data (R^2=0.524, p<0.0001 for the field and R^2=0.841, p<0.0001 for the laboratory).

The egg sac volume has a close relationship with the genital segment where it is attached. This relationship was confirmed by the results from laboratory conditions (Fig3H) where R^2=0.820 (p<0.0001) and was statistically significant in field data (R^2=0.323, p<0.001) in spite of the high dispersion (Fig.3G).

Egg packing constraint in field and laboratory conditions

If we consider that egg-sac volume is the sum of the volumes of all individual eggs plus the free volume remained in the sac, we can suppose that the packing of eggs by a female can be a function of clutch size, egg size and the constraints encountered. In ideal conditions, the egg sac volume should be greater that the clutch volume (reproductive effort) without any packing constraint.

The dispersions observed in field data, particularly in Fig.3C, could be due to the problem of packing constraint. In fact, we observed in ovigerous females of *E. affinis* three levels of egg packing. Fig 4 illustrates these situations for sacs with similar clutch sizes (between 45 to 50 eggs per clutch). The egg sac dimensions (length and width) increased when the packing constraints decrease because of the appearance of free space between eggs as shown in Fig. 4C. On the opposite situation, when the egg sac volume seems to be smaller than clutch volume produced, the eggs were piled up and tight (Fig.4A). Between these two situations, the packing of eggs can present a good geometrical distribution with more or less tightness between eggs (Fig.4B). In general, the strong packing constraint (SPC) can alter the spherical shape of the eggs. In most cases, it is difficult to distinguish visually the level of packing constraint. So, in order to quantify the packing constraint status in both field and laboratory conditions, we first plotted all individual ovigerous females data in the plane *ESv* as a function of *Cv* (Fig.5). Then, we considered two reference lines $y=x$ and $y=1.5x$. These two lines constitute the limits of the strong packing constraint (SPC) and the no packing constraint (NPC), respectively. Data plotted between the two lines are characterized by an intermediate level of packing constraint (IPC).

Both field (Fig 5A) and laboratory (Fig5B) data showed a strong linear relationship between *ESv* and *Cv* with similar and high R^2 (0.721 and 0.70, respectively). On the basis of the slope values, the *ESv* is almost double of *Cv* in all conditions (Fig.5). To identify the packing constraint, we should focus on the lower part of the data situated below the regression lines because the positively dispersed data have the highest no packing constraint (NPC). The packing constraints (IPC and SPC) were more frequent in field data (Fig.5A) compared to laboratory data (Fig.5B), especially for the SPC. The data from the different estuaries were well mixed in the regression plane (Fig5A) contrary to the laboratory data (Fig5B) where we can distinguish easily the separations between initial (in blue color palette) and final (in red color palette) experimental conditions. In laboratory conditions, the IPC and SPC are mainly represented by data from high temperature and/or salinity (Fig5B).

Then we computed the percentages of observed data in each constraint level by considering three classes SPC, IPC and NPC. Table 3 confirms that the percentage of packing constraints in the laboratory conditions (SPC and IPC classes) increased at the highest temperature of 24°C, whereas, the percentage of NPC decreased in all treatments. The higher salinity (25) compared to the optimal one (15) produced the highest percentages of packing constraints (SPC and IPC).

The distributions of the packing constraint classes, in the different estuaries, are shown in Fig 6A and they revealed clear inter-estuarine differences. In European estuaries, a north-south decreasing gradient in packing constraints appeared giving the highest values (SPC=32.35% and IPC=35.29%) in Gironde and the lowest ones in the Scheldt (SPC=2.44% and IPC=9.76%). On contrary, North American estuaries did not present clear special gradient but the lowest packing constraint percentages were noticed in the Chesapeake Bay (SPC=4.17% and IPC=8.33%) which were different from in the St Lawrence estuary sites showing a strong heterogeneity with a relatively high packing constraints.

The classification of all sampled estuaries/sites based on their relative distributions in the selected three classes of packing constraints and using Chi-square distance, gave the dendrogram of Fig 6B. This later opposed two different groups of estuaries, one composed by three sites in Europe (Elbe, Scheldt and Seine) and two sites in North America (Berthier sur mer (SL) and Chesapeake Bay) and the second group contained two sites from Europe (Loire and Gironde) and three sites from St Lawrence in North America (Isle verte, St Jean Port Joli and Montmagny). In the first group, the NPC was always higher than 60% and the SPC never reached 18% where the lowest percentage was null in the Elbe estuary. In the opposed group, the NPC never reached 50% while the SPC were always greater than 23%. The site of Montmagny (SL3) showed the lowest similarity in the second group (Fig.6B) because it contained the lowest NPC percentage in all estuaries/sites (13.33%) and the highest percentages of packing constraints SPC and IPC (33.33% and 53.33%, respectively).

In order to understand the effects of the egg packing constraints on the relationship between the *ESv* and the key reproductive traits (*Cv*, *Ev*, *Pv* and *GSw*), we selected the estuaries showing more than 10% of SPC composed by 3 estuaries in Europe (Seine, Loire and Gironde) and 4 sites in the St Lawrence estuary in North America (Table 4). Then, the correlations between *ESv* and the other selected traits and their statistical significance were calculated for each class of egg packing constraint (SPC, IPC and NPC). For all combinations, the *ESv* was highly correlated with the clutch volume (Table 4). The highest correlations were obtained for the IPC conditions (except for Montmagny (SL3)) then for SPC. On the contrary to *ESv* vs *Cv* the correlations between *ESv* and *Ev* were generally weak. In fact, in SPC situation only two estuaries (G and SL2) showed highly significant correlations (Table 4), no strong correlations were obtained for IPC conditions (one significant correlation at level 0.05) and a single estuary (Seine) with low (R^2=0.08) but highly significant correlation (+ two significant correlations for Loire and SL1 at level 0.05) for NPC. The correlation between *ESv* and *Pv* was mainly highly significant, in the case of NPC (except for Gironde and SL3, Table 4). For IPC, the relationship was highly significant for two estuaries, Loire and SL3. For SPC, in addition to Loire that was always highly correlated whatever was the constraint packing, SL2 was also highly correlated for SPC situation.

For the European selected estuaries, the correlations between *ESv* and *GSw* in the Seine and Loire populations were highly significant in case of NPC, whereas, in Gironde population this relationship was highly significant in case of IPC. No significant correlation was observed for the SPC situation. For the St Lawrence sites, excepting SL4 that showed significant correlation in IPC case, no relationship between the *ESv* with the genital segment was observed.

For the remaining estuaries (Elbe, Scheldt and Chesapeake Bay) where SPC was very low, the previous correlations in NPC were valid for *ESv* vs *Cv*. The *ESv* of ovigerous females from the Chesapeake Bay was highly correlated to the *Ev* (R^2=0.38) but showed a low correlation (R^2=0.08, $p<0.05$) with the *GSw*. On the opposed way, the other European estuaries (Elbe and Scheldt) showed high correlations ($p<0.005$) with the *GSw* (R^2=0.58 and R=0.29, respectively).

By considering all estuaries in NPC, it seems that only the European populations showed a functional relationship between the genital segment and the size of the egg sac.

Relationship between packing pressure index and clutch size

In order to understand the possible mechanisms affecting the egg packing constraints, we selected two extreme situations from the different experimentally controlled conditions. From Table 3, we identified the highest percentages of NPC in the condition of T7S15 (96.7%) which is considered as a control situation without any pressure of packing. The highest percentages of SPC were observed at the highest temperature and the highest salinity but the total number of females was relatively low per single condition (Table 3). Consequently, in order to test the relationship between *CS* and PP index in the case of SPC, the individual data were aggregated.

Figure 7 showed a significant linear relationship between *CS* and PP index with two opposed trends, a positive slope for T7S15 (i.e., NPC) condition and a negative slope in the presence of SPC. The packing constraints seemed to be independent of the clutch size. In fact, at T7S15 females was able to produce more than 80 eggs per clutch without showing any packing constraint. In contrast, for the strong packing constraint situation, even if the clutch size was smaller (maximum CS of 43 eggs) due to the high temperature and/or salinity, a negative trend still exits.

Discussion

Presence of true egg sac with external membrane in Eurytemora affinis

Our study based on a high number of detailed examinations of ovigerous females of the calanoid copepod *E. affinis* confirmed the presence of a true egg sac with external membrane enclosing the eggs. This observation was valid for 10 populations from different estuaries in

Europe and North America which increases the case studies showing the presence of an egg sac membrane among calanoid copepods (Hopkins 1977, Ohman and Townsend 1998, Kosobokova et al., 2007). This external membrane is of a great importance in the reproduction of these egg bearing copepods due to its protective and nutritive role to the eggs (Altaff and Chandran 1994).

Generally, the studies of the egg sac and its membrane are very scarce and the only one suggesting the mechanism of egg membrane formation was performed for the freshwater calanoid copepod *Helidiaptomus viduus* (Altaff and Chandran 1994). This work described the presence of an oviducal secretory gland responsible of the production of the required materials necessary to build the external sac membrane. Because *E. affinis* is a brackish species capable to live in low salinity zone of estuaries (Devreker et al., 2010) and even invade freshwater habitats (Lee et al., 2007), we can hypothesize that its egg sac membrane formation could result from similar physiological mechanisms described in *H. viduus*. The confirmation of this assumption has to be validated by further investigations. According to Altaff and Chandran (1994), the oviducal gland produces an elastic sac that envelops the oocytes until their full development then the elastic sac detaches from the oviduct bringing the eggs to the genital segment ready for internal fertilization. As soon as eggs get fertilized the elastic sac transforms to an external sac enclosing the eggs with a membrane (Altaff and Chandran 1994). In *E. affinis*, this egg sac is clearly attached to the genital urosomal segment after its release (see Fig 2).

Packing constraint in Eurytemora affinis populations

Most likely, the internal origin of the egg sac composed by the membrane and a fixed number of spherical eggs requires accurate scheduled steps that *E. affinis* female has to optimize. In fact, when the external membrane reached the last steps before extrusion, only its size and elasticity will delimit its maximum volume. However, this elasticity should have some limit and the female will end the reproductive cycle by packing the eggs into the sac. This last step may lead to packing constraints when the reproductive effort (number of eggs per clutch x egg volume) is much higher than the maximal capacity (volume) of the external sac.

In order to track these situations in *E. affinis*, we suggested the use of a simple index called Packing Pressure Index (PP index) that is the difference between the final egg sac volume and the reproductive effort (clutch volume). This index and the reference lines of the packing constraints (see Fig. 5) permit the separation of the different patterns of packing constraints which are SPC, IPC and NPC. Contrary to the clear separation between European and North American populations of *E. affinis* based on the trade-offs of their reproductive effort (Chapter II, part 2), the packing constraints analyses separated two groups of estuaries by mixing trans-Atlantic populations (Fig 6). The first group of estuaries composed by the southern populations in Europe (Gironde and Loire) and the northern ones in St Lawrence estuary (Isle verte, St Jean Port Joli and Montmagny) was characterized by a high SPC and/or IPC cases. The second group, showing the highest percentages of NPC, assembled the southern site in St Lawrence (Bertier sur Mer), the Chesapeake Bay representing the most southern estuary in this study and three northern European estuaries (Seine, Scheldt and Elbe). Our results underlined that the packing constraint situation of *E. affinis* ovigerous females was primarily dependent on the geographical location of the estuary rather than the genetic similarities (North American clades vs European clade). Indeed, in Europe a clear positive north-south gradient in the distribution of the packing constraint cases was observed with the highest SPC and NPC in southern and northern estuaries, respectively. In North American estuaries considered in this paper, the gradient in packing constraint cases appeared to be opposed to the European’s one. But one should not forget that the St Lawrence estuary and the Chesapeake Bay are much bigger than the European estuaries and globally contrasted on their packing constraints situations.

The experimental study based on a representative population from the Seine estuary revealed that the SPC and IPC situations increased with the increase of temperature and/or salinity. This finding comforts the observed north-south gradient in Europe that may not reflect only a thermal gradient but also a clear difference in the habitat’s quality of *E. affinis*. The contrasted situations between Gironde and Scheldt observed in this study (Fig. 6) reinforced this statement. In fact, Gironde is one of the most turbid estuaries situated in the southern part of Europe where ovigerous females of *E. affinis* presented a small size and low egg production particularly these recent years (David et al., 2007). Whereas, the Scheldt estuary presented a suitable habitat for *E. affinis* (with larger females and higher clutch sizes) particularly, after the recent restoration actions improving the water quality (Mialet et al., 2010). In North American estuaries, the observed differences in the repartition of the packing constraint classes in *E. affinis* ovigerous females, especially for St Lawrence sites that could lead to the highly variable habitats regarding the salinity gradient (Winkler et al., 2008). The 4 sites considered in this study contain one salt marshe (Isle verte) and three sites affected differently by the tidal regime where two clades of *E. affinis* could co-occur (Winkler et al., 2008). Ovigerous females of *E. affinis* sampled in Montmagny site showed the highest constraints in egg packing which was opposed to the site of Berthier sur Mer in spite of their close locations in the oligohaline zone. In addition to the genetic heterogeneity between *E. affinis* populations in the St Lawrence estuary (Winkler et al. 2008), the differences between habitats as pointed out by the packing constraint analysis should be considered. The packing constraint pattern in the ovigerous females from the Chesapeake Bay were similar to those of the Scheldt estuary indicating that it is a favorable habitat for *E. affinis*.

Effect of the packing constraint on the relationship between ESv and key morphological and reproductive traits

The reproductive effort (i.e. *Cv*) and the *ESv* were positively correlated to the body size of *E. affinis* ovigerous female from both field and laboratory conditions (Fig. 3). In general the correlations between *ESv* and *Cv* were highly significant in all packing constraint situations with the highest R^2 often observed in IPC situations (see Table 4). Therefore, we can consider the *ESv* as the proxy of the reproductive effort of egg bearing copepods that can be easily measured. The other relationships between *ESv* and morphological traits were influenced by the packing constraint situation. For example, the *ESv* and *Pv* were better correlated in NPC situations, which suggested that the reproductive energy (leading to a clutch and a sac) was well scaled with female size in this situation. The experimental results using *E. affinis* from the Seine estuary revealed that the *ESv* is linearly related to the genital segment width (Fig 3H). When all data from the field were combined, this relationship was significant but with a high dispersion due principally to the North American populations (see Fig. 3G) that did not show any significant correlation when considred alone (R^2=0.14; p=0.12). In fact, when only European populations were used, a strong correlation was obtained (R^2=0.70; p<0.0001). Therefore, this relationship between *ESv* and *GSw* seem to be valid only for European populations in NPC situation. Gironde was the only estuary where the correlation between *ESv* and *GSw* was only significant in IPC situation (Table 4). Moreover, Gironde was the only European population showing a strong correlation between *ESv* and *Ev* in SPC condition (R^2=0.62; p<0.0001). This relationship was also valid in NPC situations for Scheldt, Seine and Loire in Europe and for Isle verte and Chesapeake Bay in North America. Beck and Beck (2005) used a numerical algorithm to optimise the quantity of eggs that could be packed in a body cavity of a given shape and size. They hypothesized that the relationship between allocation to reproduction (clutch size times maximum egg size) and the egg volume should be positively linearly related in log-log plot with or without packing constraints. We showed in this study that *E. affinis* can exhibit a range of packing constraint situations and therefore we can not assign it to a single reproductive strategy (with or without packing constraints). In absolute terms, all egg bearing copepods should face the packing constraints because the reproductive effort could not evolve independently of the egg size (Beck and Beck 2005). But the positive relationship between *ESv* and *Ev* in SPC situations were only observed in Gironde and St Jean Port Joli populations. For the European case this is probably due to the low number of eggs per clutch.

Importance of egg sac in egg bearing copepods: generalities with egg packing constraints

To our knowledge, our study is the first one that analysed thoroughly the packing constraints in a single species (of species complex) of copepods. The conclusions of this study confirmed that the packing constraints in copepods occur and could have an ecological meaning. The relationship between the reproductive effort, the packing constraints and egg volume should be carefully addressed in future studies. In fact, the egg sac is of great importance in the life history of egg bearing copepods being involved in the survival of the species by insuring the egg carrying (Logerwell and Ohman 1999) but also can increase the vulnerability to predation (Mahjoub et al., in press). Therefore egg bearing copepods facing these opposed selection pressures evolved to produce either a single or two egg sacs that differ in shape and in position (Logerwell and Ohman 1999). The egg packing constraints, ignored in most copepod studies, could reflect the copepod responses to their environmental conditions. The only study considering egg packing constraints in copepods was performed by Beck and Beck (2005). But the database used (originated from Caley et al. (2001) paper which in turn mostly based on Poulin's work (Poulin 1995)) contained a compiled data set on free living (broadcast spawners and egg bearing) and parasitic copepods. Moreover, the high dispersion in their data sets made it difficult to generalize their application to copepods. However, we believe that the packing constraints algorithm (Beck & Beck 2005) could be adapted to copepods but at lower taxonomic levels with ecologically similar species.

Ecological significance of packing constraint situations

The packing constraints observed in the field were much higher than those observed under controlled conditions where the high number of external factors that could affect the packing constraints could not be accurately controlled. Consequently, we considered two opposed situations in the experiments to compare the SPC and the NPC by plotting clutch size as a function of the PP index (see Fig. 7). In the T7S15 condition where we found the highest percentage of NPC, the relation with the clutch size followed a positive trend meaning that the greater is the clutch size the more the PP index increase positively (i.e. increase the NPC situation). In contrast, the case of SPC that we observed at salinity 25 and temperature 24°C, the clutch size was negatively correlated with the PP index which means that the greater was the clutch size the higher was the constraint. These patterns could be explained by the fact that at the lower temperature the fecundity and the survival were high (Chapter I, part 2) and the growth was slow (Chapter I, part 1), so the egg sac size always matched to the clutch size produced. Whereas, at stressful conditions, when the female invest more energy in her survival (Chapter I, part 2), the fecundity was usually lower. Indeed, when the female produced bigger clutch size compared to the "average situation", packing pressure appeared because the egg sac size remained constant. This suggests that the reproductive effort and the *ESv* are not usually in equilibrium. The female can receive an environmental signal that allows her to prepare a certain reproductive effort but during egg lying the female can face some perturbations (i.e. from different sources of stress) that may favor the appearance of packing constraints. Because the allocation of the reproductive effort could be subdivided into a number of eggs and individual egg size (Smith and Fretwell 1974), we don't know exactly the link of egg sac formation steps (i.e. membrane and eggs) and the decision of reproductive effort partition. The external egg sac carried by ovigerous female resulted from two internal reproductive mechanisms leading to simultaneous or sequential steps of membrane and egg clutch (oocyte maturation, egg fertilization and their release) formations. The quantity of material used to produce the elastic internal sac should be correlated to the clutch size in order to avoid any disequilibrium between the number of ready fertilized eggs to be extruded and the carrying capacity of the membrane (without reaching the limit of its elasticity). In the situation when the equilibrium between eggs'clutch and membrane sizes was reached, NPC was expected. However, for any internal or external reasons giving higher egg production approaching the limit of the distortion capacity of the elastic membrane, the packing constraint pressure is enhanced and the eggs appeared to be piled up and tight in the sac. In SPC, it seems that a clear desynchronism between the signal leading to the egg membrane formation and the allocation of reproductive effort. In such cases, it is interesting to understand the determinism of these phenomena.

These different egg packing constraint situations can be observed in the field and can indicate some external sources of stress or perturbation of the ovigerous females' reproductive cycle. This study showed that the packing constraints were site/estuary specific and did not show any similarity to the genetic structure of the considered populations/clades, but concerned the environment faced by *E. affinis* populations.

Our results suggest that the packing constraints analysis could be used as an indicator of the habitat quality of *E. affinis* populations within and between different estuaries. This hypothesis could be tested experimentally by exposing different populations of *E. affinis* to several sources of external perturbations.

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Tables

Table 1. Sampling sites of *E. affinis* with the latitudes, the dates and the number of observations.

Continent	Estuary		Latitude – Longitude	Dates (Nbr of observation)				
				2006	**2007**	**2008**	**2009**	**Other years**
Europe	**Elbe**		53°32’06N - 09°47’31E	17 Mar (20)				
	Scheldt		51°21’06N - 04°14’58E	4 Apr. (19)	11 Apr. (11)	6 Apr. (11)		
	Seine		49°28’33N - 00°27’54W	23 May (19)	10 Oct (21) - 6 Nov (16)	8 Mar (19) - 10 Apr (27) 8 Jun (34) - 8 Jul (21) 20 Sept (11) - 4 Nov (15)	25 Feb. (20)	10 Feb. 2010 (18)
	Loire		47°17’23N - 02°01’52W	18 Apr. (20)	15 Nov. (31)	16 Apr. (9)	25 Jun (16)	
	Gironde		45°14’80N - 00°43’50W	20 Apr. (20)			15 Apr. (15)	
North America	**St Lawrence**	**Isle Verte**	48°00’20N - 69°25’50W		18 May (15) - 12 Jun (20) 11 Jul (26)		22 May (7)	
		St Jean Port Joli	47°12’59N - 70°16’22W		11 Jul (6)	15 Jul (19)	20 May (20) 2 Jun (33)	
		Montmagny	46°59’26N - 70°33’13W		14 Jun (20)		May (7)	
		Berthier sur Mer	46°56’07N - 70°44’07W		11 Jul (1)	15 Jul (17)	29 May (5)	
	Chesapeake Bay		39°23.81'N - 76°03.32'W			16 Apr (10) - 15 Dec (19)	6 Feb (22) - 28 Apr (11)	May 2002 (6) Apr 2003 (4)

Table 2: Exposition of the different experimental conditions of *E affinis* culture in the laboratory with the number of generation and the number of total ovigerous female observed at each experimental condition

Initial experimental conditions				**Final experimental condition**			
Temperature	**Salinity**	**Nbr of generation**	**Total Nbr of observations**	**Temperature**	**Salinity**	**Nbr of generation**	**Total Nbr of observations**
7°C	15	4	121	24°C	15	5	112
20°C	15	8	205	24°C	15	5	196
20°C	25	7	182	24°C	25	5	135

Table 3: Percentages of the different case of packing in the different laboratory condition culture of *E. affinis*

Packing constraint situations	**Init. cond.**	**Fin. cond.**	**Init. cond.**	**Fin. cond.**	**Init. cond.**	**Fin. cond.**
	T7S15	**T24 S15**	**20TS15**	**T24S15**	**T20S25**	**T24S25**
Strong packing constraint (%)	1.6	3.4	0.5	2	4.3	6.9
Intermediate packing constraint (%)	1.6	20.7	10.2	14	12.9	32.4
No packing constraint (%)	96.7	75.9	89.3	84.0	82.8	60.7

Table 4: Table showing the relationships with the egg sac for estuaries that present more than 10% of strong packing constraint

Continent		**Europe**			**(North) America**			
estuaries		**S**	**L**	**G**	**SL1**	**SL2**	**SL3**	**SL4**
ESV vs CV	SPC	**0.88**	**0.90**	**0.95**	**0.67**	**0.76**	**0.96**	**0.97**
	IPC	**0.98**	**0.96**	**0.996**	**0.98**	**0.97**	**0.88**	**0.97**
	NPC	**0.86**	**0.87**	**0.83**	**0.87**	**0.86**	-	**0.53**
ESV vs EV	SPC	10^{-5}	0.03	**0.62**	0.07	**0.73**	0.35	0.21
	IPC	0.04	0.01	0.003	0.27	0.01	0.13	0.54
	NPC	**0.08**	0.15	0.25	0.34	0.05	-	0.18
ESV vs PV	SPC	0.06	**0.53**	0.004	0.21	**0.60**	0.71	0.22
	IPC	0.10	**0.70**	0.21	**0.65**	0.10	0.14	0.49
	NPC	**0.44**	**0.73**	0.12	**0.66**	**0.13**	0.13	0.27
ESV vs GSW	SPC	0.06	0.15	0.007	0.31	0.33	0.002	0.03
	IPC	0.20	0.06	**0.52**	0.15	0.26	0.04	**0.94**
	NPC	**0.38**	**0.32**	0.04	0.11	0.05	0.19	0.04
SPC (%)		14.65	25.00	32.35	25.00	23.00	17.39	33.33
IPC (%)		21.21	28.13	35.29	36.11	27.66	53.33	21.74
NPC (%)		64.14	46.88	32.35	38.89	48.94	13.33	60.87

Figures

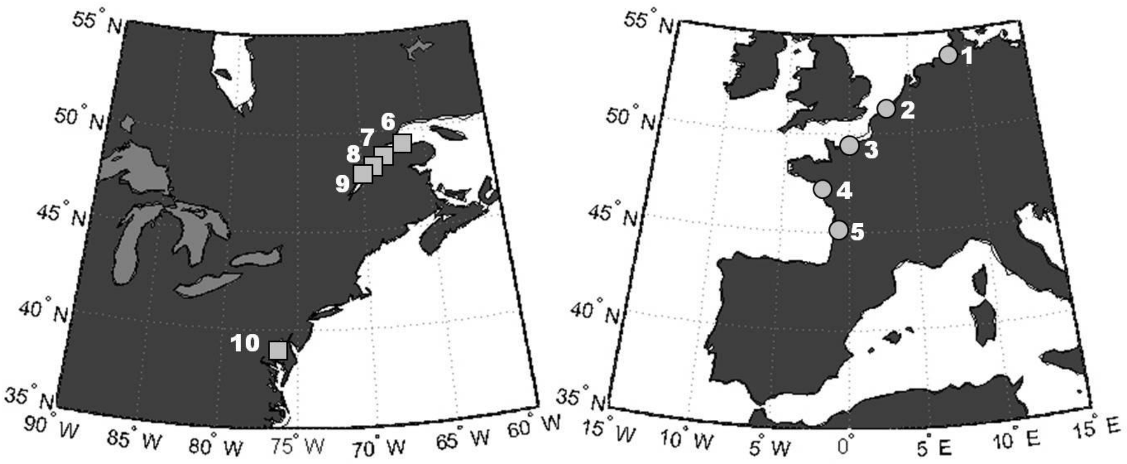

Figure 1. Map of the sampling locations in Europe (1: Elbe, 2: Scheldt, 3: Seine, 4: Loire and 5: Gironde) and North America (St Lawrence estuary sites: 6: Isle Verte, 7: St Jean Port Joli, 8: Montmagny and 9: Berthier sur Mer; and 10: Chesapeake Bay)

Figure 2: Lateral view photo of the egg sac of *E. affinis* illustrating the membrane (grey arrows showing visible parts of the membrane) and the attachment point to the genital urosomal segment (black arrow) (scale=200µm).

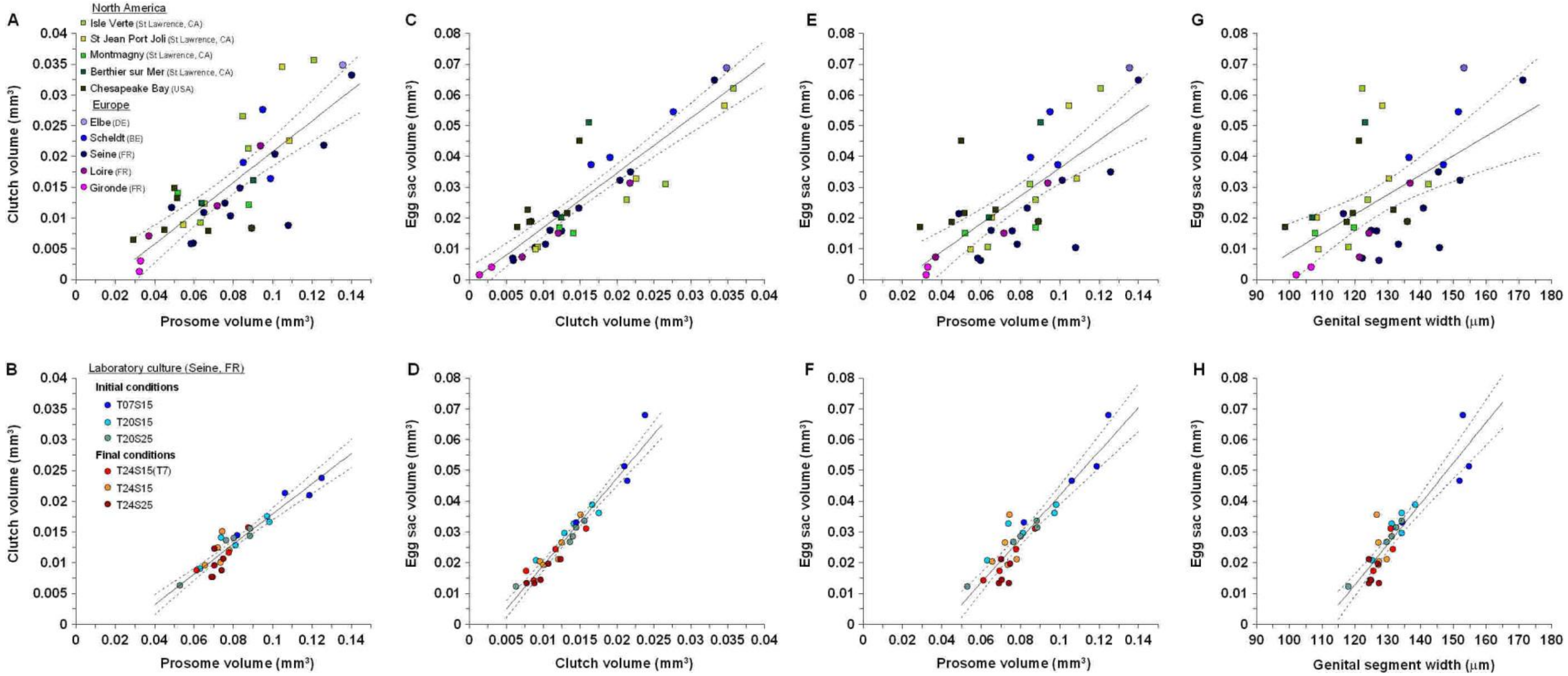

Figure 3 : (A, B, C, D): relationship of the reproductive effort (clutch volume) with the prosome volume and the egg sac volume. (E, F, G, H): relationship of the egg sac volume with prosome volume and the genital segment width. A, C, E, G: represent the graphs of the data from the different estuaries. B, D, F, H: correspond to the graphs of the data obtained from the laboratory.

The continuous line is the fitting and the dashed lines are the 95% interval of confidence of the fitting.

Figure 4. Photos of the different cases of egg sac packing in *Eurytemora affinis*'s ovigerous females. The photo on the top shows the case of strong packing constraint of the eggs in the sac. The photo in the middle shows the intermediate packing constraint with a good geometrical disposition of the eggs. The photo on the bottom illustrates the no packing constraint with clear space between eggs. (Scale=200µm)

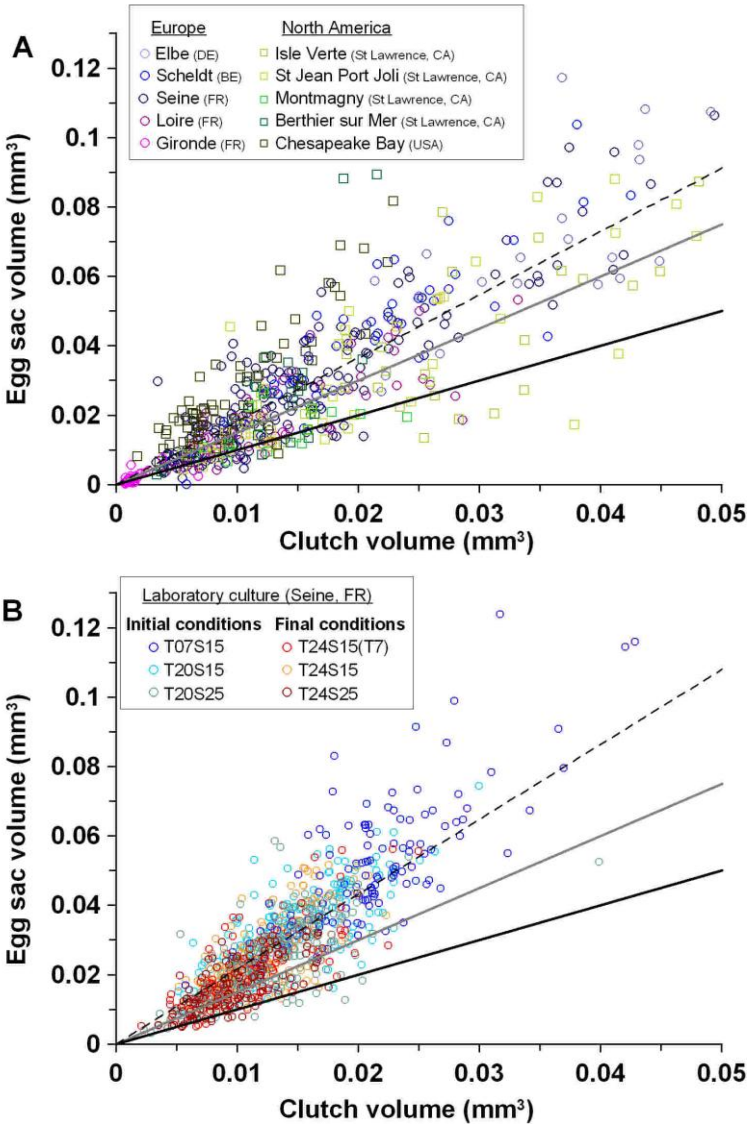

Figure 5. Relationship between the reproductive effort (clutch volume) and the egg sac volume for each individual ovigerous female from the field (A) and the laboratory (B). The dashed lines are the linear fits for different estuaries data (R^2=0.721, y=1.823x) and for laboratory data (R2=0.70, y=2.159x). The continuous lines delimit the different egg packing constraint cases. The black continuous line ($y=x$) shows the limit of the strong packing constraint, the grey line ($y=1.5x$) is the limit of the no packing constraint. The interval between the two continuous lines contains the intermediate level of packing constraint. The rectangles contain the labels of each population used from in situ and laboratory conditions.

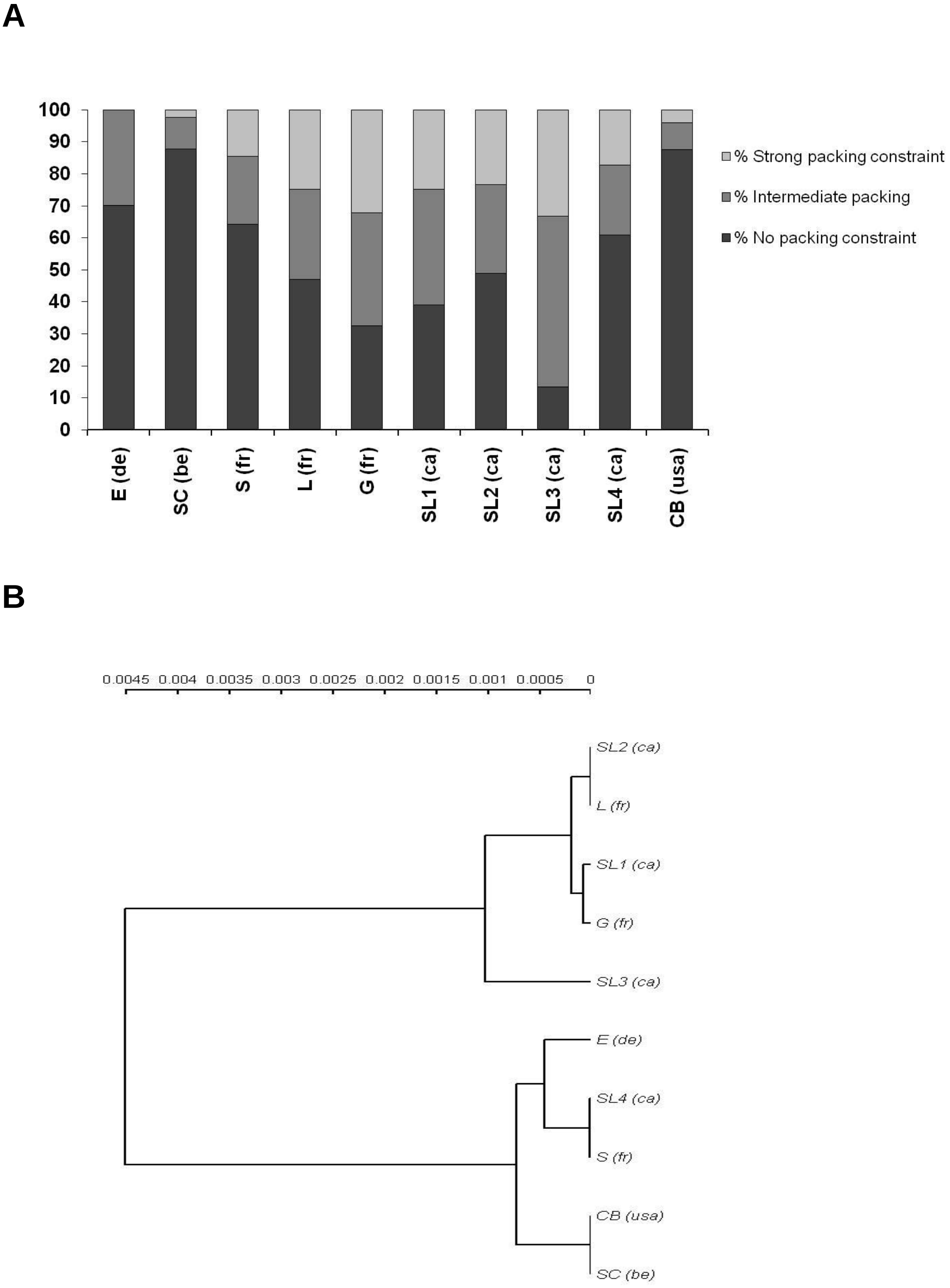

Figure 6. (**A**)Percentages of the different packing states in the egg sac of *E. affinis* from different estuaries (E (de): Elbe (DE), SC (be): Scheldt(BE), S (fr): Seine (FR), L(fr): Loire(FR), G(fr): Gironde(FR), SL1(ca): Isle Verte (St Lawrence, CA), SL2(ca): Saint Jean Port Joli (St Lawrence, CA), SL3(ca): Montmagny (St Lawrence, CA), SL4(ca): Berthier sur Mer (St Lawrence, CA) and CB(usa): Chesapeake Bay (USA)), (**B**) classification of the different sites/estuarieson the basis of the Chi-square distance of the proportion of packing constraint classes.

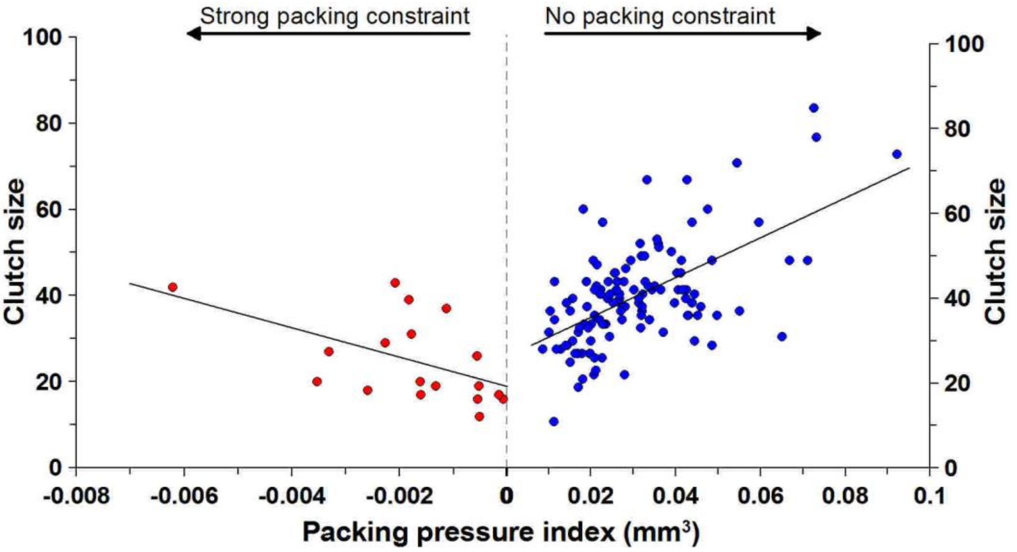

Figure 7. Relationship of the clutch size with the packing pressure index in two contrasting conditions (strong packing constraint and no packing constraint). The blue labels represent the data from the highest % of no packing constraint (T7S15 condition) and the red labels symbolize the data from the highest % of strong packing constraint (T24 and S25 conditions). The continuous lines are the linear fits.

Chapter III: **Behavioral reproductive strategy in *Eurytemora affinis*: contribution of female and male**

Introduction

Because the reproduction is the key biological and evolutionary process in all living organisms, its different steps have been deeply studied in most biological groups. In animals and particularly in sexually reproducing ones, the formation of a fertile diploid zygote requires copulation between a male and a female. Consequently, the role of each sex in determining the success of the offspring and ensuring the preservation of the species is crucial. Females get more attention in most studies related to the reproduction and the role of males in the reproductive strategy received less attention (Titelman et al. 2007). In more recent studies, mostly based on terrestrial biological groups such as insects, the contribution of male in reproduction becames increasingly clear showing the role of paternal fertility (i.e., ejaculate size) in the reproductive success or by supplying females with nutrients that can increase egg production and/or improve female's somatic growth (Fischer et al., 2009, Wedell 2005). The consideration of both male and female reproductive traits with their genetic correlations can influence sexual selection (Stearns 1992). Fischer et al. (2009) demonstrated a genetic correlation between female (egg size) and male (spermatophore mass) reproductive traits in a butterfly. Furthermore, females of many invertebrates exhibited a polyandrous mating behaviour which could be beneficial for increasing offspring viability or avoiding the negative effect of genetic incompatibility (Tregenza and Wedell, 2002; Price et al., 2008). These recent studies confirmed, at least in crickets and drosophila (fly), that polyandrous mating behaviour is not necessarily a wasting energy strategy as has been admitted in several groups including copepods (Lucks 1937, Katona 1975).

Wedell et al., (2002) reviewed the recent evidences of the implication of males in the reproductive strategy and confirmed that males are sensitive to the risk of sperm competition and can adjust their ejaculate size which explains their spatial and temporal variability. In addition, they showed that males can assess mating status and relative fecundity of females.

In recent “horizons” section paper, Titelman et al. (2007) discussed the status of the mating strategy in copepods, by underlying the persistent gaps in the understanding of whether copepod mate by chance or by active selection. Unfortunately, the mating mechanisms in copepods, which similarly to most animals, involving a variety of competitive and selective stages (Wedell et al., 2002) were not deeply studied. Most papers described the pre-copulatory behavior that could be complex and involving the capacity of males to locate females by using both mechanical and chemical cues (Titelman 2007; Kiørboe and Bagøien, 2005; Katona 1973, 1975). The studies of the energy allocation in copepod reproduction were generally unbalanced from two points of views. First, the majority of studies converted the reproductive energy of females in terms of specific egg production rates (i.e., proportion of carbon invested in the clutch per unit of time) neglecting the trade-off between the size and the number of eggs (see Chapter II). Second, little attention has been given to the reproductive investment of the male in terms of spermatophore production and morphological features (Titelman et al., 2007). However, an accurate review of the literature dealing with copepod reproduction revealed that some evidences of mate selection were documented in few examples of freshwater calanoid copepods (Chow-Fraser and Maly, 1988; van Leeuwen and Maly, 1991; Watras 1983; Berger and Maier 2001). All these papers confirmed that males of most freshwater calanoids studied are capable to recognize receptive mating females. Consequently, males showed a preference to mate with mature and gravid females ready to produce a clutch. The male was able to assess not only the external sexual maturity of the adult female but also her reproductive status reflected by the condition of her oviduct (dark phase = gravid; clear phase=non gravid) (Watras and Haney, 1980). This could explain why males of *Eurytemora affinis* were able to differentiate between immature females (C5 stage) and adult females that were experimentally sorted as gravid females (see Katona 1975). Copepod females, in this reproductive stage, seem to produce pheromones to enhance the probability of fertilization success. These chemicals, supposed to be sexual pheromones by Katona (1973) allowed *E. affinis* males to clearly discriminate conspecific males and other congener females (i.e. *E. herdmani*). Katona (1973) concluded that the existence of sex-pheromones in *E. affinis* confirmed by the high percentage of successful searches is not principally due to vibration or water currents or random encounter but it is an oriented mate seeking. These pioneer conclusions made by Katona in the early 70s have been widely cited in the followed studies without deep investigations in the mate choice in copepods. The literature review also pointed out an artificial separation between the freshwater studies confirming the role of the physiological reproductive status of females (Watras 1983) and marine studies dealing with swimming behavioural patterns (Titelman et al 2007). In both areas of research, the studies of male reproductive system are still scarce. The most documented measures of size distributions of copepod spermatophores concerned the deep and large marine copepods, mostly of genus *Euchaeta* (Hopkins and Machin, 1977; Mauchline 1994). Recently, Ali et al. (2009) studied the morphology of females and the attached spermatophores in the wild caught calanoid copepod *Eudiaptomus graciloides*. They tried to relate the number of spermatophores carried by a female to its size, considering the hypothesis that multiple mating is useless in the reproduction of copepods. However the number of spermatophores attached to a female could be affected by the number of males and the sex-ratio in general. In fact, Hopkins and Machin (1977) showed that the seasonal variations in the mean number of spermatophores per *Euchaeta norvegica* female were associated to the proportion of males in the adult population. It is thus difficult to link the number of spermatophores attached to a copepod female by only using behavioural hypotheses (e.g. the escape capability of the female from a seeking male). More recent study by Ceballos and Kiørboe (2010) based on experimental study with the free spawning copepod *Acartia tonsa* suggested a mate choice based on size-oriented sexual selection. It is clear that the experimental studies are more appropriate to identify the mechanisms involved in reproductive strategy, because in the field the high variability of the environment could biases the observed patterns. For example, it is difficult to understand the reasons of the under-dispersed distribution (mean-variance plot) of the number of spermatophores per female of the copepod *Euchaeta norvegica* reported by Hopkins and Machin (1977). Spermatophores production rates of *E. affinis* (Choi and Kimmerer 2009) and of *Acartia tonsa* (Ceballos and Kiørboe, 2010) were estimated in the laboratory by using an artificial unbalanced sex-ratio. However the sex-ratio and the experimental manipulations of individual copepods leading to their isolation in small groups could limit the generalization of the observed conclusions (Brown and Shine, 2009). We suppose that mate choice can occur in calanoid copepods but its identification need adequate experimental protocols.

In the previous chapter, we already confirmed the role of female reproductive strategy by applying the optimal offspring size theory. In this chapter, we question about the roles played by each sex of *E. affinis* in the reproductive strategy. We attempt to clarify the mating selection strategy and its consequence on the fitness of each sex as well as their offspring.

Material and method

Experimental protocol

The copepod culture of *E. affinis* originated from the Seine estuary is the same used in the previous chapters. For this study, we used the same initial (T7S15, T20S15 and T20S25) and final (T24(7)S15, T24S15 and T24S25) experimental conditions.

From each generation of each experimental condition, between 20 to 40 ovigerous females and 20 males were sorted to measure their body sizes and their reproductive traits. For each ovigerous female, 5 to 15 eggs per sac were measured and the number of attached spermatophores was counted (Table1). Then, we differentiated between used and unused spermatophores as shown in figure 1 illustrating two opposed states of spermatophores with filled one and an almost empty one. Contrary to eggs, spermatophores have a fragile structure that could be damaged by manipulation, so they were measured directly on the female when they were entirely visible. When it was possible, all attached spermatophores were measured for every female. All the measurements were realized using ImageJ software.

Morphological measurements

Individual body sizes were estimated for both males and females, by measuring prosome length and width and calculating the prosome volume by assuming an ellipsoid shape (see Chapter II). The eggs were spherical and the images allowed measuring accurately their area and deducing their equivalent diameter and volume. For spermatophore measurements, the simple assumption of the ellipsoid shape (that needs only two simple measures), as admitted by Ceballos and Kiørboe (2010) for *Acartia tonsa*, was not appropriate in the case of *E. affinis*. Consequently, we examined the general morphological structure of a spermatophore and defined 4 morphological parts as shown in Figure 2. The spermatophore usually described as a flask tube in copepods showed in *E. affinis* a more complex structure showing a clear separation between an upper transparent part and a basal opaque part that prolonged by a tube (the spermatophore neck). We measured first the spermatophore length (SP_L) and width (SP_W) (Figure 2). Then, in order to estimate accurately the total volume of a spermatophore, we followed these steps:

i) The basal part showed a conical shape (a in Fig. 2) and its volume was given by equation (1)

$$V_{con} = \frac{\pi}{3} r^2 h_{con} \tag{1}$$

where V_{con} is the volume of the cone (the basal part), r is the radius ($r = \frac{1}{2} SP_W$) and h_{con} is the height (the length of the basal part).

ii) The main volume of the spermatophore is composed by a cylindrical tube (b in Fig.2) ended by a semispherical cap (c in Fig. 2).

The volume of the cylindrical part was calculated as follows

$$V_{cyl} = \pi r^2 h_{cyl} \qquad (2)$$

Where V_{cyl} is the volume of the cylindrical tube (middle part), r is the radius (as in equation (1)) and h_{cyl} is the height (the length of the middle part)

The volume of the semispherical cap was calculated by equation. (3):

$$V_{cap} = \frac{\pi}{3} h_{cap}^2 \left(3r - h_{cap}\right) \qquad (3)$$

where V_{cap} is the volume of the semispherical cap (extremity of the spermatophore), h_{cap} is the height and r is the radius as in equations (1) and (2).

iii) The total volume of the spermatophore (V_{SP}) is the sum of the three subparts ((1), (2) and (3)):

$$V_{SP} = V_{con} + V_{cyl} + V_{cap} \qquad (4)$$

The visible spermatic part lodged in the upper section of the spermatophore (delimited by arrows in Fig.2) gives an idea on the level of sperm use by the female (see examples in Fig. 1). The volume of this intern spermatic part (V_{int}) was estimated in two steps as in equations (2) and (3) and used to calculate the quantity of sperm (V_{sperm})

$$V_{sperm} = \left(V_{cyl} + V_{cap}\right) - V_{\text{int}} \qquad (5)$$

This allowed to the estimate the percentage of sperm used. Because in unused spermatophore we often noticed a small free space between the internal spermatic part and the external envelope, we fixed a threshold value of 10% of the sperm used that discriminate the used spermatophores (values >10%) from the unused ones (≤10%). This last step allowed the counting of used spermatophores by the female.

Statistical analyses

The distributions of the number of spermatophores per female were calculated, for each experimental condition, then fitted with the Poisson probability density function using curve fitting toolbox of Matlab software.

The relationships between reproductive and/or morphological traits were assessed by calculating Pearson correlation coefficient. When the correlation was statistically significant a simple linear regression between the independent and dependent variables was performed.

In these treatments, either individual data or average data per generation were used. Finally, the average values of the considered traits per experimental condition were calculated and compared, by using the value of each generation as a replicate.

Results

Distribution of the mean number of spermatophores per female: effect of experimental conditions

Figure 3 shows a plot of the mean number of spermatophores per female per generation in all experimental conditions with the corresponding variances. We deliberately removed the last generation (fifth) at the condition T24S15 that represented a clear outlier with a high over-dispersion situation with an observed mean of 2.8 spermatophores per female and an associated variance of 6.52. This analysis shows that all experimental conditions are symmetrically distributed around the reference line ($\sigma^2 = \mu$) corresponding to the Poisson probability distribution (the dashed line). We can not discriminate between experimental conditions by using this simple dispersion mean-variance analysis. The linear regression passing through the origin was highly significant and explained around 53% of the total variance in mean-variance relationship. In spite of the relatively high dispersion of the experimental data around the regression line (continuous) which was almost superimposed to the reference line (dashed), we can justify the assumption that the number of spermatophores carried by a female followed the Poisson probability distribution. Consequently, we aggregated the data per experimental treatment and calculated the observed probability distribution functions (PDFs) in each experimental condition. For the experimental condition T24S15, all observed females including those from the last generation (removed in Fig. 3) were considered. Figure 4 shows that these distributions were well fitted by a Poisson's probability function. Nonetheless, the distribution of spermatophore number in the initial conditions (well acclimated conditions) followed perfectly the Poisson probability function (all R^2 values were greater than 0.99). On the contrary, in the final conditions, where the mean of the spermatophore number and its associated variance increased, the best fit of Poisson PDF was obtained for T24S25 (R^2=0.990), then in T24S15 (R^2=0.971) and finally in T24(7)S15 (R^2=0.942). These results could be associated to the stressful and transitional regime after temperature increase. The increase of the mean number of spermatophore was accompanied by a higher variance leading to think that under stressful conditions the equilibrium in the mating process was disrupted.

By considering that the number of spermatophores attached to *E. affinis* female is an indication of the mating intensity, Table 2 shows clearly an increase of this activity with the temperature (by comparing T7S15, T20S15 and T24S15). We can also notice that the stressful conditions (high salinity or extreme heat shock) decreased the mating activity. Because the number of spermatophores as an integrator of the mating success in *E. affinis* involves several mechanisms, we should first compare its relationship to the sex-ratio.

Effects of sex-ratio and female reproductive condition on the mean number of spermatophores per female

Figure 5A shows the relationship between the mean number of spermatophores per female and the sex-ratio (male:female) calculated for each generation. This analysis clearly demonstrated a positive and significant linear trend meaning that when the number of adult males was relatively higher than the number of females, the mean number of spermatophores attached to female increased. This pattern can be explained by the increase of the encounter rate because the experimental volume was kept constant in our study. The highest value of the sex-ratio of 2.7 was observed for one generation (F6) at T7S15 and corresponded to a mean number of spermatophores of 1.2. In contrast, the high mean number of spermatophores observed at T20S15 of 1.4 was obtained for a sex-ratio in favour of males (1.33). The differences in the slopes of both linear regressions (Fig. 5A) were principally due to the temperature values that enhanced the mating activity at 20°C compared to 7°C. When the temperature was increased to 24°C, the mean number of spermatophores increased with high dispersion in final conditions (values ranged between 0.33 and 2.8) but the sex-ratio remained globally stable in each final condition. We can mention that the extreme heat shock in the final condition T24(7)S15 induced a high dispersion and the positive trend observed in the initial condition T7S15 became invalid. On the contrary, the combined two other final conditions (T24S15 and T24S25) showed a statistically positive trend but less significant than observed in the initial conditions (Fig. 5B). If we consider only temperature effect at the optimal salinity of 15, the mean number of spermatophores per female showed a positive trend with temperature (Table 2). However, the variance associated to the mean number of spermatophores tended to increase showing a high individual variability between females.

This pattern could be due to the stressful conditions that might disturb the mating processes but it could also be explained by a non random process of mating choice.

The second hypothesis suggests that the transfer of spermatophores which is the last step of the mating, was realized with a high discrimination between individual females leading to the slightly over-dispersion of the data at the final conditions T24S15 and T24(7)S15 (see Table 2). In order to test this hypothesis, the reproductive condition of each female was considered. We separated between gravid (dark oviduct) and non gravid (clear oviduct) females reflecting their sexual maturity. Figure 6A shows that excepting the condition T7S15 with 43.33% of gravid females all other conditions showed a percentage of gravid females greater than 50% and reaching 68% in T24S15. Globally in all experimental conditions, *E. affinis* females were more gravid than non gravid. Figure 6B clearly showed a preferential attachment of spermatophores on gravid females in all experimental conditions. In fact the mean number of spermatophores attached to gravid females was almost 3 times higher than in non gravid females.

Consequences of females and males fitness and reproductive investments on the population of E. affinis

Figure 7A demonstrated that the observed mean sex-ratio is related to the daily mortality rate. In fact, the lowest mortality rate observed at T7S15 was accompanied by the highest sex-ratio. However, the transfer of individuals from the low temperature initial condition to 24°C induced a high mortality (3 times higher) leading to equilibrate the sex-ratio around 1. This means that the differences in mortality between sexes could explain this pattern. The conditions T20S15 and T24S15 were the most stable conditions for both traits (Fig. 7A). The high salinity of 25 and the transfer to 24°C induced an increase in both mortality rate and sex-ratio. This analysis confirmed the necessity to consider both life cycle traits (sex-ratio and mortality rate) in the interpretation of the distribution of spermatophores number detailed in the previous figures. Figure 7B showed that in addition to the variability in the mean number of attached spermatophores, the percentage of used spermatophores by the female also varied. When each couple of initial and final conditions was considered separately a same trend was observed corresponding to a simultaneous increase in both number and percentage of use in the attached spermatophores. We also noticed that the highest percentages of used spermatophores were observed in individuals acclimated initially at low temperature (T7S15 and T24(7)S15). When the sperm use by a female was expressed as quantity (volume of sperm) and its percentage compared to the total sperm available in all spermatophores attached, a different pattern was obtained (Fig. 7C). The differences between initial and final conditions were notable for individuals that were acclimated to high temperature compared to those acclimated to 7°C. The sperm quantity and percentage were very low at T20S25. In general, the females in all conditions used less than half of the total quantity of sperm available. When the mean volume of an individual spermatophore was considered for each condition, we noticed that the highest value ($0.52\ 10^{-3}\ \mu l$) was obtained at T7S15 whereas, for the other conditions, the values were globally stable below $0.4\ 10^{-3}\ \mu l$ (Fig. 7D). If we compare this pattern to the mean egg volume produced in all conditions, we noticed that the values were close and globally stable between initial and final conditions showing the highest values for the eggs produced by females acclimated to the lower temperature (T7S15 and T24(7)S15).

Reproductive investment in both male and female of E. affinis: size effect

Figure 8A showed a highly significant positive linear trend between male prosome length and the spermatophore length. This means that larger males produced bigger spermatophores. A similar relationship between mean egg diameter and female prosome lentgh was also obtained (Fig. 8B). We can observe a higher dispersion in the case of female's relationship compared to the male case. In all experimental conditions, the mean prosome lengths of males and females were positively correlated. The linear regression of Fig. 8C explained almost 86% of the total observed variance. This means that in each experimental condition and each generation, the growth of both sexes was similarly affected. On the contrary to Fig. 8C, when the reproductive investment traits (egg diameter vs spermatophore length) were regressed only 13% of the variance was explained (Fig. 8D). This uncoupling between adult sizes, which were strongly related and their reproductive traits which were poorly related, confirmed the role of the trade-off in the reproductive strategy.

Because of the strong relationship between the mean values of female and male body sizes, we tried to explore what can be the relationship between individual female prosome length and the average size of the spermatophores she carry. Figure 9A showed a sifgnificant positive linear trend between female prosome and spermatophore lengths. This means that bigger spermatophores were attached to larger females. In addition to this positive relationship, we found that the quantity of sperm used by a female was proportional to the number of attached spermatophores (Fig. 9B). This means that the female can adjust the use of the quantity of sperm to the number of spermatophors attached that were linked to the intensity of mating behaviour.

Discussion

Spermatophore attachment in E. affinis

During mating, *E. affinis* male seek for a female oriented by a chemical diffusion, as described by Katona (1973). Then, when the encounter between mates is successful and the female did not exhibit an escape behavior (i.e., she is ready for copulation), the sexual activity is engaged including to two major steps. The first step is the seizure of the female by the geniculate right antennule. Then, both mates adopt a copulation position on dorsal side with the male on the top holding in the tip of his left fifth leg a ready spermatophore (as in Fig. 10, right photo) to be attached to the gonopore of the female situated on the ventral side of its genital urosomal segment (Fig. 10, left photo). In our study, all spermatophores were well attached to the gonopore and reinforced by sticky cement like substance. In spite of the high number of observations in our study, we never found a detached spermatophore contrary to what was reported by Choi and Kimmerer (2009) for *E. affinis* from San Francisco estuary. The absence of detached spermatophores could be due to the experimental protocol designed in our study to reduce all sources of stress including those due to frequent manipulations (i.e., several observations per day). Moreover, when the sex-ratio was artificially unbalanced in favor of females, males may increase their mating tentative and this could result on the possible loss of spermatophores. We considered here that the number of spermatophores can be used as a good descriptor of the different successive steps involved in the mating of *E. affinis*. A high number of spermatophores attached to a female could be considered as an indirect measure of the intensity of the reproductive activity and particularly the frequency of mating that resulted. Because only the first clutch was considered in our study, we avoided any confusion that could be introduced by the effect of the age of the female.

Mating strategy in E. affinis effect of sex-ratio and experimental conditions

In our study, the number of spermatophores carried by a female was fitted by a Poisson distribution. We confirmed experimentally that during initial conditions, when individuals were acclimated to stable and constant conditions for several generations, the number of spermatophores was modeled as a Poisson process. However, when temperature increased to

24°C, the variance was greater than the mean of the number of spermatophores suggesting an over-dispersion. The only available study on the dispersion of the mean-variance of spermatophore number was realized in the field for the big marine calanoid copepod *Euchaeta norvegica* (Hopkins and Machin, 1977). The mean number of spermatophores observed in this species and the low proportion of males in the field could partly explain the surprising under dispersion published by Hopkins and Machin (1977). In our study, the sex-ratio varied greatly allowing testing its effect on the number of attached spermatophores. Kiørboe (2007) used the data from Hopkins (1982) and confirmed that the fraction of fertilized females of *E. norvegica* (carrying at least one spermatophore) was nonlinearily related to the sex-ratio (male:female). We showed in this chapter that not only the fraction of fertized females (data not shown here) but also the mean number of spermatophores was linearilly related to the sex-ratio in the initial conditions. This confirms that the number of spermatophores is a good estimator of the mating activity in an egg bearing copepod as shown for *E. affinis*. Moreover, when the experimental conditions were stresfull or the copepods did not have enough time (in terms of number of generations) to acclimate to the high temperature, a high variability in the mean number of spermatophores and its associated variance was observed. The use of the multi-generational protocol allowed testing the consequences of the sex-ratio produced by a given generation (the same cohort) on the mating process that was involved in the contribution to the following generation. The sex-ratio was the best independent variable that explained the patterns of the number of spermatophores. When we tried to check whether the number of spermatophores was a function of the densities of males and females produced in each generation, the results were not statistically significant (the results are not shown here). This could be explained by the fact that the experimental volume used (2 liters) was not limiting for the encounter or mating process. In fact, at 15°C, Choi and Kimmerer (2009) estimated a volume of search rate for *E. affinis* males of 7.2 ±4 l h^{-1}. Even if this search volume is very high, by considering its lower limit (i.e., 3.2 l h^{-1}), we can conclude that the encounter between males and females of *E. affinis* were very frequent in our experiments.

In order to compare our laboratory results to field conditions, several samples of *E. affinis* were examined to check the distribution of the attached spermatophores number. Without presenting these data, we found that the mean number of spermatophores in *E. affinis* in the Seine estuary was comprised from 0.33 and 2.85 spermatophores.$female^{-1}$ over a total of 380 ovigerous females checked. The highest means of the spermatophores number were associated to higher variance leading to a global over-dispersed distribution. This observation confirms that the mating frequency is very high in the field and that the observed unbalanced sex-ratios in favor of males (Devreker et al., 2008; 2010) is characteristic of this population of *E. affinis* from the Seine estuary. We already showed (Chapter I, part 2) that the sex-ratio of *E. affinis* from the Seine population differed significantly from those observed in the same laboratory conditions for two other populations from the Gironde and the St Lawrence estuaries. It seemed that the low mortality rate, particularly for males, explained this pattern because an increase of mortality often affected negatively males and changed the sex-ratio. But the exact mechanisms of sex-determination in *E. affinis* (in copepods in general) still unclear (Souissi et al. 2010). We can only suppose that having too many males in a population of *E. affinis* would guarantee a successful mating at all seasons, even during critical periods of low densities (Choi and Kimmerer, 2009).

Advantages of multiple mating in E. affinis

Kiørboe (2007) separated the copepods in two classes, the first one was supposed to be composed by species where males are assumed to have an unlimited mating capacity and females need to be mated just once. The second class is composed by species that need multiple successive mating, where a re-mating is required for each clutch. Our observations, go above this simplistic separation of two opposed classes and suggest that several intermediate situations can occur in copepods especially for *E. affinis*. In fact, Katona (1975) already confirmed that the presence of several spermatophores in a single female of *E. affinis* was common and suggested that the origin of spermatophores comes from a series of mating with different males rather than from a single prolonged mating. Choi and Kimmerer (2009) showed that the maximal number of spermatophores produced by *E. affinis* males during 24 hours was 3. Because the duration of the mating in *E. affinis* was very short and generally did not exceed several minutes (Katona 1975, our observations), we can suppose that *E. affinis* females carrying more than 1 spermatophore were mated several times with different males. This conclusion was true at least for the females carrying high numbers of attached spermatophores observed in this study (>3 in Fig. 11, the maximum number observed was 14). In his experiments, Katona (1975) tested the hypothesis made by Heinle (1970) stating that *E. affinis* females required copulation to fertilize each clutch of eggs and did not lay eggs in the absence of males. Even thought, Katona (1975) used very low temperatures (2°C and 4.5°C) that were not very representative of the natural thermal range in the field, he observed two successive clutches produced by fertilized females in absence of males (Katona 1975).

But he concluded that re-mating is required in *E. affinis* even if he reported that some isolated females were able to produce nauplii after 38 and 57 days of isolation. Unfortunately, Katona (1975) did not explore the mechanisms of sperm storage in *E. affinis*. Barthélemy et al. (1998) on the basis of the study by Gaudy and Pagano (1987), suggested that *Eurytemora velox* the congeneric species of *E. affnis* did not have a seminal receptacle and thus could not store internally the sperm. If we admit that *E. affinis* could not store internally the sperm, we can question about the advantage of the multiple mating.

We showed, for the first time in this study, that *E. affinis* females originated from the Seine estuary can use several spermatophores. Moreover, we confirmed that the total quantity of sperm used by a female was linearly linked to the number of attached spermatophores (see Fig. 9B). In addition, the percentage of the quantity of sperm used by a female was less than 50% in all experimental conditions (see Fig. 7C). This leaded us to hypothesize that *E. affinis* was able to utilize sperm content from more than one attached spermatophore. Our results contrasted with all other empirical observations in *E. affinis* (Katona 1975) and more generally in copepods stating that only one spermatophore (i.e., one male) can be used by a female to fertilize her clutch. However, recent studies in other invertebrate groups (such as insects), confirmed the advantages of polyandrous mating behavior (Tregenza and Wedell, 2002). In copepods, to our knowledge, the only published work confirming the existence of polyandry was performed by Todd et al. (2005) in the ectoparasitic copepod *Lepeophtheirus salmonis*. They used genetic markers to confirm the multiple paternity of a single clutch in this copepod species supposed to follow the same general admitted rule of genetic material (sperm) use from a single male. Our study strongly suggests that polyandry is rather common in *E. affinis* population (at least from the Seine estuary). The advantages of such strategy for increasing female and offspring fitness were largely discussed in terrestrial groups (Fischer et al. 2006; Tregenza and Wedell, 2002; Price et al. 2008) and completely ignored in copepods (Titelman et al. 2007).

Role of each sex in the reproductive strategy of E. affinis

The role of male in copepod reproductive strategy did not receive much attention. Consequently, the role of the male has been assigned to simply mating and transfer of the spermatophore to the female. The asymmetrical parental investment in copepods was largely admitted (Titelman et al. 2007). But, the contribution of each sex in terms of ecological and evolutionary strategies has never been addressed in copepods. On contrary, only the quantity of energy allocated by females to produce clutches was considered. Regarding female's reproductive strategy, we already demonstrated that the existence of trade-off between egg size and number occur in laboratory and field populations of *E. affinis* (Chapter II). Even though copepod reproduction was well documented in females, the detailed role of their contribution in the reproductive performance was less studied compared to other terrestrial groups (i.e., insects). Furthermore, the situation is worst for males where its role was only limited to behavioural copulatory processes. For examples, the number of studies describing the spermatophore size in copepods was very rare (Hopkins 1978, Blades and Youngbluth 1981, Mauchline 1994; Ali et al. 2009; Ceballos and Kiorboe 2010). The analysis of these publications led to suggest that the spermatophore size is related to the copepod body size. Mauchline (1994) showed a bimodal distribution of spermatophore size in *E. norvegica* without providing the clear mechanisms of this pattern. But he suggested that males can adjust the size of their spermatophores depending on the body size of the species and to the model depth. Ali et al. (2009) found, for an average prosome length of the freshwater calanoid *Eudiaptomus graciloides* of 790 µm, the spermatophore length was 326 µm (43.3% of the female body size). In our study, the spermatophore length of *E. affinis* was on average of 358 µm corresponding to 40.3% of female prosome length. However, the comparisons with the photo provided by Katona (1975) showing a female with 20 attached spermatophores revealed that they were relatively small (almost 30% of female prosome length). We can speculate that the investment of males in their output could be variable from one population to another and from an experimental condition to another. Moreover, the spermatophore size could be species specific for example the supplementary material of Ceballos and Kiørboe (2010) showed that the spermatophore size of the free spawning *Acartia tonsa* was very small. This explains why the mean spermatophore volume measured in our study was 10 to 100 times bigger than those produced by *A. tonsa*.

In our study, the correlation between female and spermatophore sizes suggested that *E. affinis* males were able to produce spermatophores having an "optimal" size from female's point of view. Another possibility to explain this pattern is the selection applied by females during mating process to choose only large males capable to produce large spermatophores (Ceballos and Kiørboe 2010). In our study, we found a significant correlation between female and male sizes and their respective reproductive outputs (egg and spermatophore sizes). The genetic correlations between male and female traits are poorly documented. Fischer et al. (2009) found such pattern in a butterfly and reported that males provided larger females with bigger spermatophores and more fertile sperm, indicating that males may be excursing mate choice during copulation. The sperm can be used to improve the fitness of both female and offspring in a butterfly (Fischer et al. 2006), we suspect that *E. affinis* was able to take advantage of multiple mating and also from the total quantity of sperm available. When males are abundant as in the case of *E. affinis* in the Seine estuary, this could offer an advantage that was not discussed in early studies assuming that males have a minor role in the reproductive strategy of copepods.

Evidence of mate choice and sexual selection in E. affinis

Our study confirmed that both sexes play complementary roles in the reproductive strategy of *E. affinis*. The mating processes in copepods were mostly described from behavioural angle (Titelman et al. 2007). The description of the different behavioral patterns observed in the cryptic mating of copepods could only confirm the complexity of the pre- and post-copulatory mechanisms in copepods. In addition, the reproductive structure in both sexes was described from pure morphological point of view (Barthélémy et al. 1998; Blades and Youngbluth 1981; Corni et al. 2001). Consequently, our understanding of the mate choice and/or mate selection in copepods remained unclear. Recently, Cabellos and Kiørboe (2010) reported the first evidence of sexual selection by mate choice in the copepod *A. tonsa*. They suggested that the selection is based only on the size with a preference of mating with larger partners. In a freshwater calanoid copepod, Ali et al. (2009) did not find a strong relationship between female body length and the number of spermatophores. We showed in our study that the number of spermatophores is rather linked to the sex-ratio and that the hypothesis made by Ali et al. (2009), assuming that larger fertilized females could easily avoid multiple mating due to their swimming capacities, was not justified. In fact, the multiple mating observed in *E. affinis* could occur in other egg-bearing copepods. Moreover, the mate choice in copepods has been documented in freshwater copepods. In fact, Watras and Haney (1980) already described the oscillation between two reproductive conditions in *Diaptomus leptopus* and their link to egg-clutch production. These reproductive conditions corresponded to the gravid phase manifested by dark oviduct and non gravid phase corresponding to clear oviduct. This means that the oviduct in gravid females contained oocytes ready for fertilization. Consequently, it is logical to assume that this pre-clutch extrusion phase is critical and the fertilization is mostly needed during this phase in order to avoid waste of unfertilized gametes. Watras (1983) was the first that suggested the existence of a discriminatory mechanism in mate location based on the reproductive condition of a female in a diaptomid copepod.

Chow-Fraser and Maly (1988) confirmed that two diaptomid species discriminated between gravid and non gravid females, whereas, another species (*Epischura*) did not discriminate between these two reproductive phases but mated exclusively with virgins of a specific relative size. This study clearly confirmed the divergence in the mating strategy between copepods needing re-mating (i.e., in two diaptomid species) and *Epischura* that required only a single mating for producing several successive eggs. Moreover, Chow-Fraser and Maly (1988) already mentioned that mate recognition could involve both chemical and morphological cues. The chemical cues hypothesis in diaptomids was tested and confirmed by van Leewen and Maly (1991) in laboratory using behavioural observations. They also showed that the percentage of females with spermatophores was much higher in gravid females compared to non gravid ones in 4 diaptomid species from the field. Berger and Maier (2001) also confirmed that mating and spetmatophore placement in *Eudiaptomus gracilis* usually occurred with gravid females rather than non gravid ones (85% of spermatophores attached to gravid females).

Katona (1975) performed his copulatory behavioural experiments in *E. affinis* using exclusively gravid females ("…an adult female with a ripe (darkened oravy).") but he did not compare the effect of the reproductive condition. He only mentioned that males did not mate with immature copepods (i.e. juveniles). Our study confirmed that the mean number of spermatophores carried by gravid females of *E. affinis* was much higher than the one carried by a non-gravid female (see Fig. 6B). This makes evidence of a clear choice during the mating process. Males can assess the reproductive state of gravid females that were more receptive to copulation. Consequently, the male increases his probability of encountering a gravid female without wasting time and energy on searching a non-reproductive female (van Leewen and Maly 1991). It seems that the same mechanism described earlier in freshwater diaptomids can apply to the estuarine copepod *E. affinis*. Moreover, we made the first evidence of possible advantage of polyandry in the copepod *E. affinis* because females were able to benefit from their mate choices (with a single or multiple partners). This suggests that sexual selection, such as mating with the preferred males could play an important role not only for improving fitness compounds but also for selecting good genes (Taylor et al. 2008 and references therein).

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Tables

Table 1. Number of individuals from both sexes measured in each condition with the numbers of individual eggs and spermatophores measured. The total number of spermatophores attached to females was indicated between parentheses.

Condition	# of males	# of females	# of eggs measured	# of spermatophores measured
T07S15	80	161	1175	44 (116)
T20S15	100	246	1694	66 (214)
T20S25	100	200	1481	40 (120)
T24(7)S15	100	148	941	50 (199)
T24S15	100	200	1892	104 (345)
T24S25	100	158	1156	54 (167)
Total	580	1113	8339	358 (1161)

Table 2. Mean number and its variance of spermatophores attached to females in each experimental condition. The fitted values of Poissons's probability distributions (see Figure 4) and the associated parameters of goodness of fit (R² and RMSE) were shown.

Condition	Observed mean	Observed variance	λ	R^2	RMSE
T07S15	0.727	0.962	0.691 ± 0.028	0.9975	0.0076
T20S15	1.346	1.386	1.316 ± 0.040	0.9971	0.0063
T20S25	0.865	1.052	0.793 ± 0.026	0.9980	0.0063
T24(7)S15	1.199	1.494	0.999 ± 0.145	0.9416	0.0291
T24S15	1.725	2.854	1.496 ± 0.122	0.9714	0.0174
T24S25	1.057	1.111	1.038 ± 0.065	0.990	0.0127

Figures

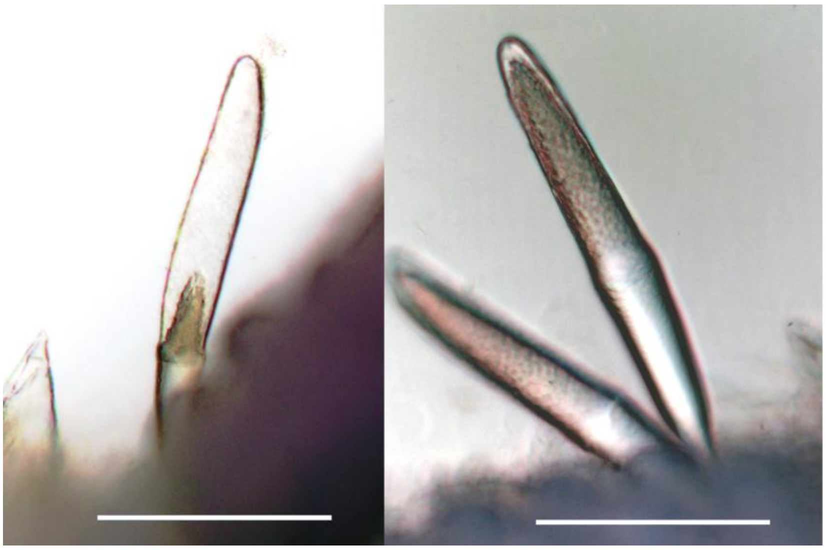

Figure 1. Photos of attached spermatophors in *E. affinis* females illustrating two opposed status: entire unused spermatophore (right) and almost empty spermatophore with used spermatic part (left). Horizontal white bars are 200µm scale.

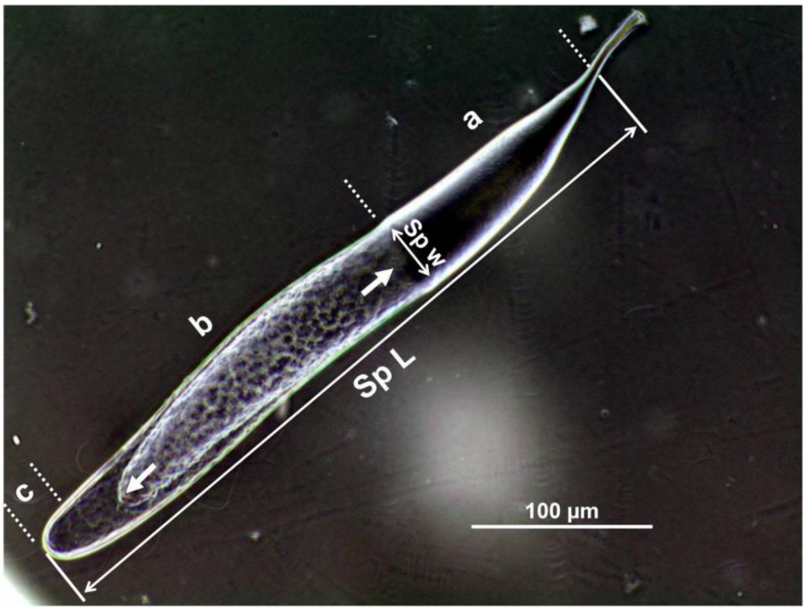

Figure 2. Detailed morphology of a detached spermatophore of E. affinis. The different measured parts (delimited by the dashed small lines) are: the basal part with a conic shape (a), the cylindrical tube (b) and the semispherical cap shape in the extremity (c). The internal spermatic visible part is delimited by the white arrows. Total length of the spermatophore (Sp L) was delimited by the double arrows line. The position where the external spermatophore width (Sp w) was measured is indicated by the small double arrow.

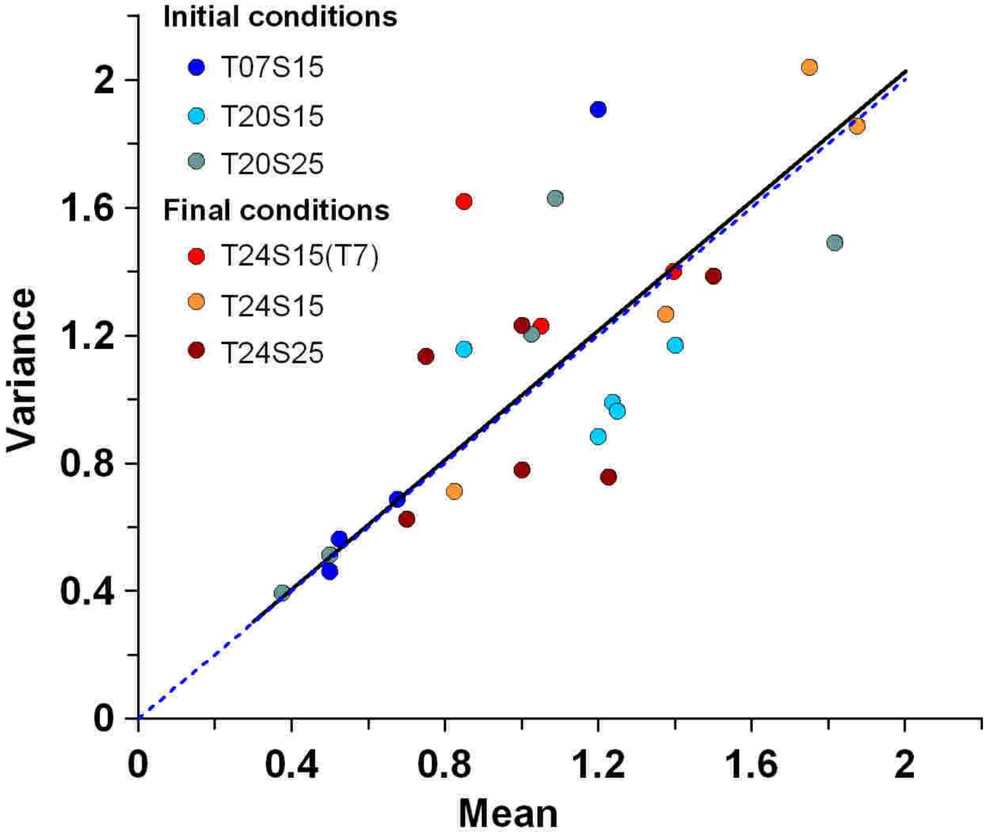

Figure 3. The mean variance relationship for the number of spermatophores per female of *E. affinis*. The linear regression equation (y=ax) fitted to the data is y=1.017x (R^2=0.529, $p< 0.001$). The dashed line ($\sigma^2 = \mu$) shows the position of a characteristic Poisson distribution. The colors of the circle symbols designate the different generations considered in each condition.

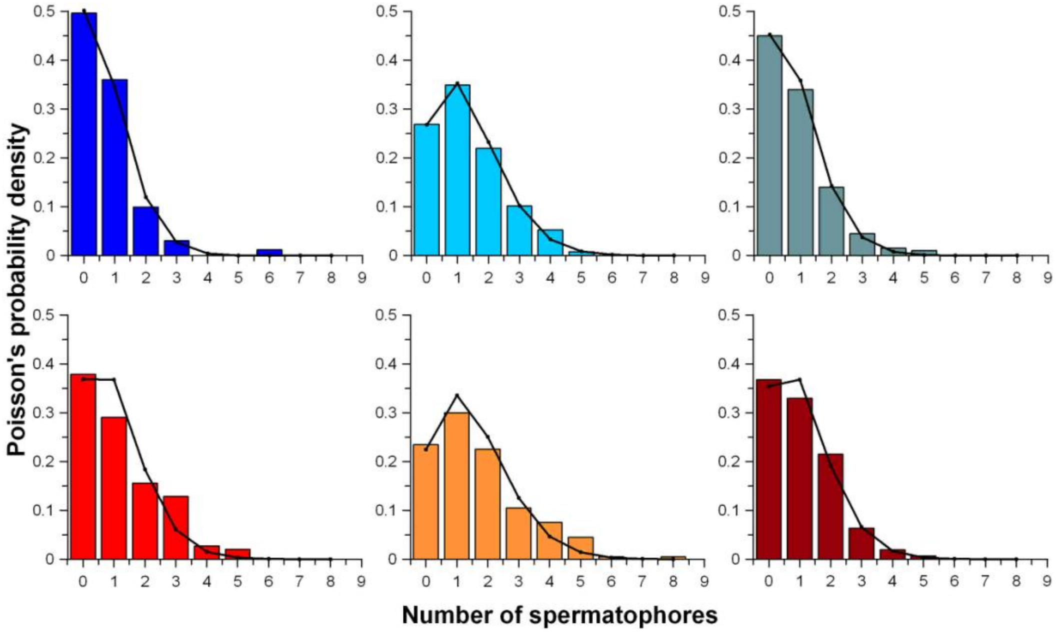

Figure 4. Bar charts of observed distributions of the number of spermatophores per female, and their fitting curves (continuous lines and symbols) to the Poisson's probability distribution in the 6 experimental conditions differentiated by colors (the colors as in Figure 3 correspond to initial conditions on the top and final condition in the bottom).

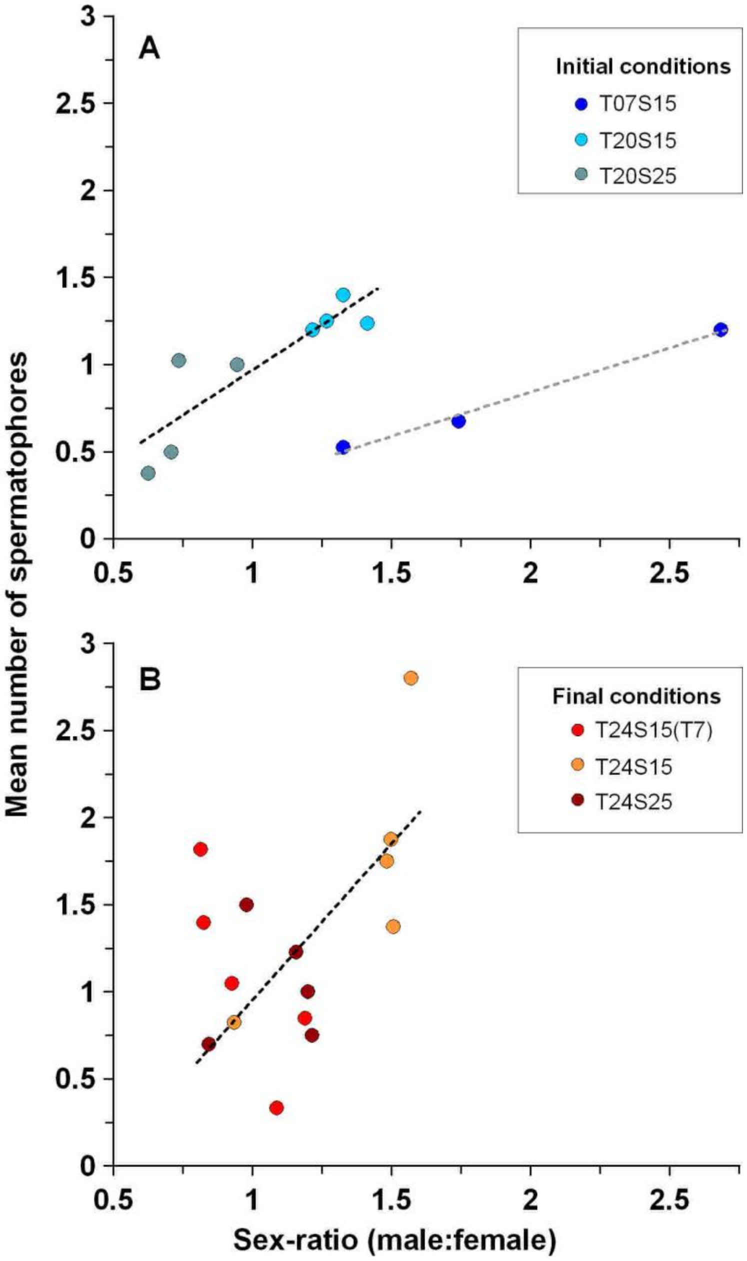

Figure 5. Relationship between the mean number of spermatophores per female and the sex ratio (male:female) observed in each generation in the initial (A) and final (B) conditions. The dashed black lines corresponded to the statistically significant linear regressions in combined data from T20S15 and T20S25 (in A) and their final conditions (T24S15 and T24S25 in B). The dashed grey line represents the positive trend of the initial condition T7S15.

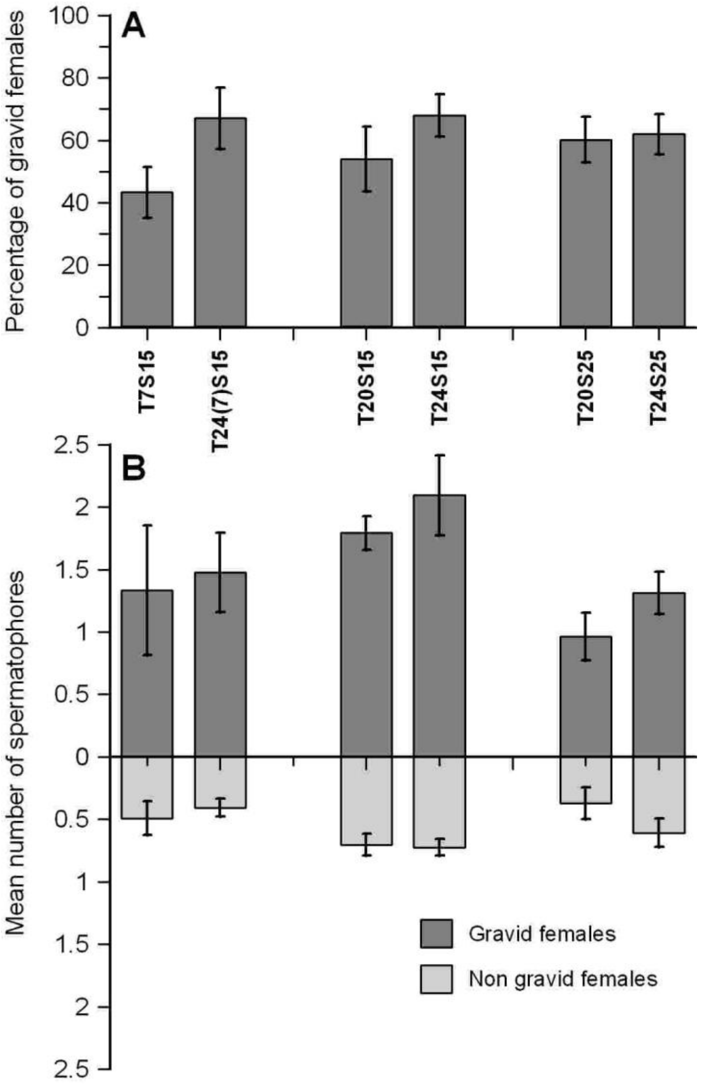

Figure 6. A: variability of the gravid females' percentage in each experimental condition. B: opposed bar charts of the mean number of spermatophores in gravid (upper panel) and non gravid (lower panel) females in each experimental condition. Vertical bars are the standard errors representing the inter-generations variability.

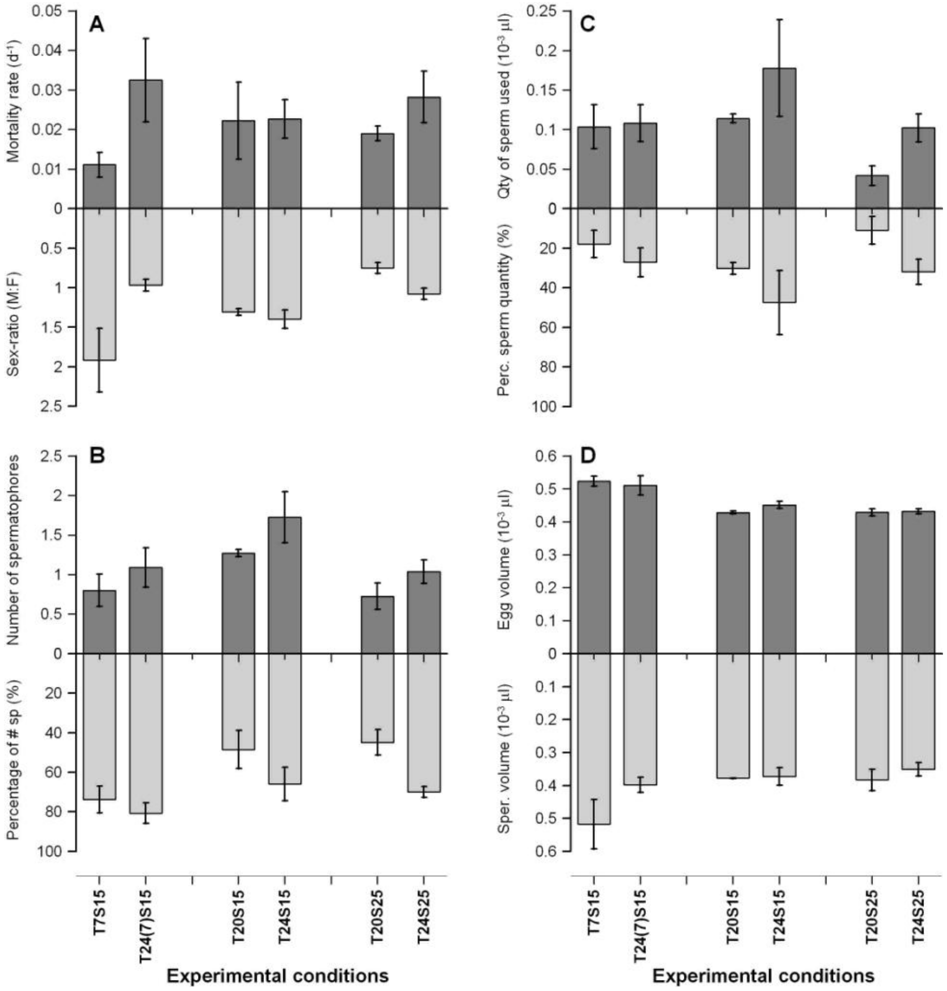

Figure 7. Variability in all experimental conditions of the mean values of: A: daily mortality rate (d^{-1}) and the sex-ratio (M:F). B: the number of spermatophores and the percentage of used spermatophores. C: the quantity of sperm used and its percentage. D: the egg volume compared to spermatophore volume. Vertical bars are the standard errors representing the inter-generations variability.

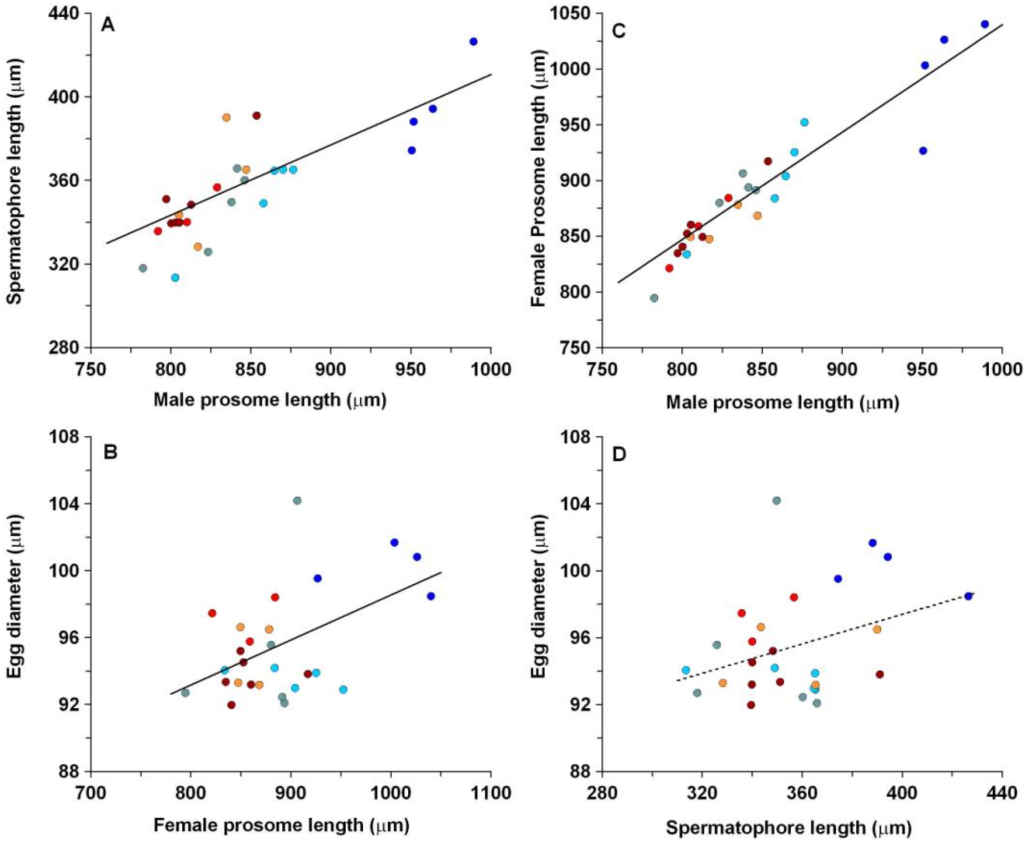

Figure 8. A: Effect of male prosome length on spermatophore length and the linear regression line ($y= 0.337x + 73.852$; $R^2=0.55$). B: Effect of female prosome length on egg diameter and the regression line ($y = 0.027x + 71.548$; $R^2=0.257$). C: Relationship between male and female prosome lengths and the linear regression line ($y = 0.963x + 76.463$; $R^2=0.858$). D: Relationship between spermatophore length and egg diameter with the dashed linear regression corresponding to a low significant level ($R^2=0.13$; $p<0.05$). The colors of the different symbols designated the generations of each experimental conditions as in Figure 3.

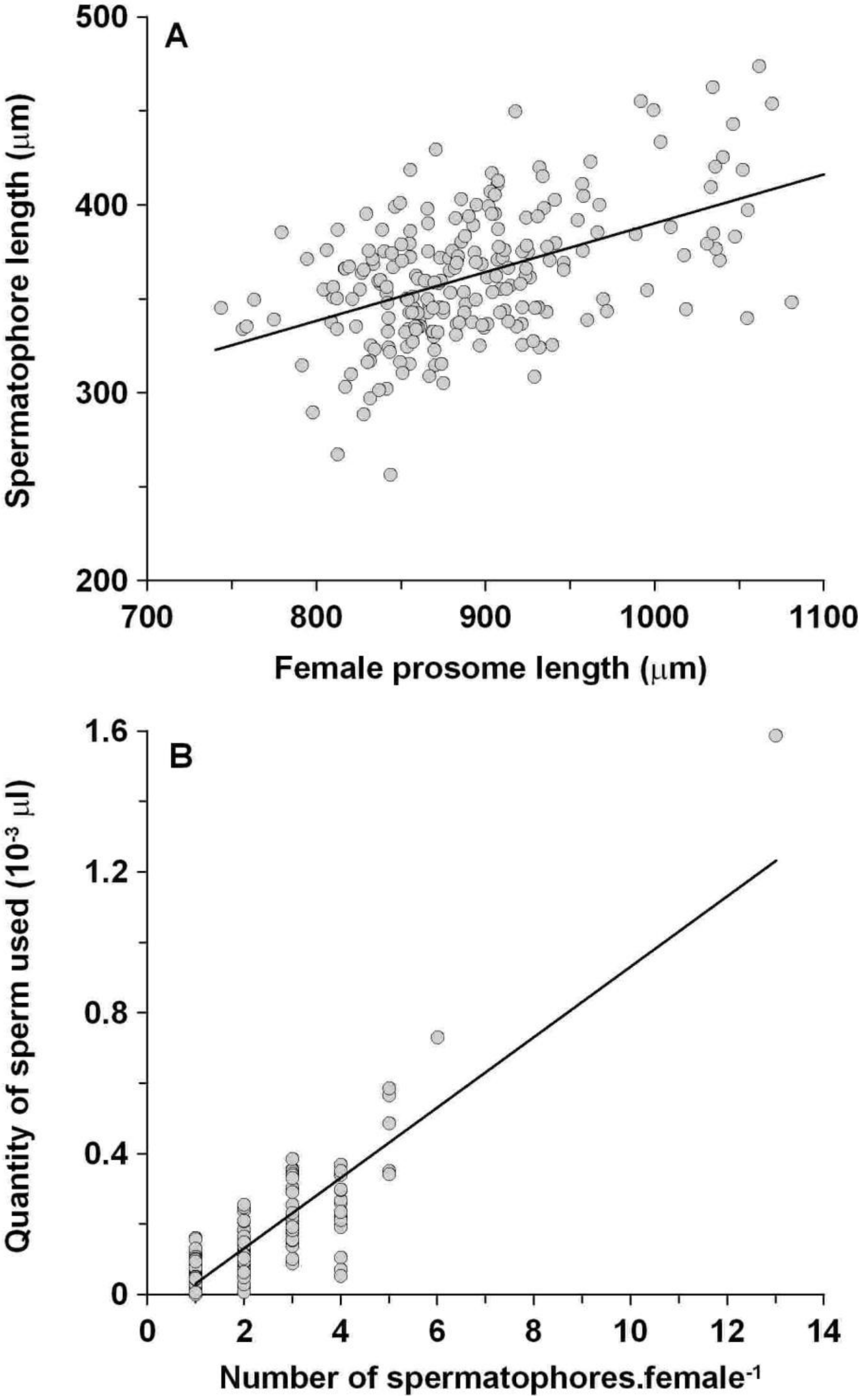

Figure 9. A: Relationship between prosome length and the mean spermatophore length in each individual ovigerous female from the laboratory (all conditions combined). The continuous line corresponds to the linear regression $y = 0.26x + 130.45$ ($R^2=0.242$; $p<0.001$). B: Relationship between the number of spermatophores and the the quantity of sperm used in each individual female and their linear regression line of $y=0.1x+0.07$ ($R^2=0.80$; $p<0.001$).

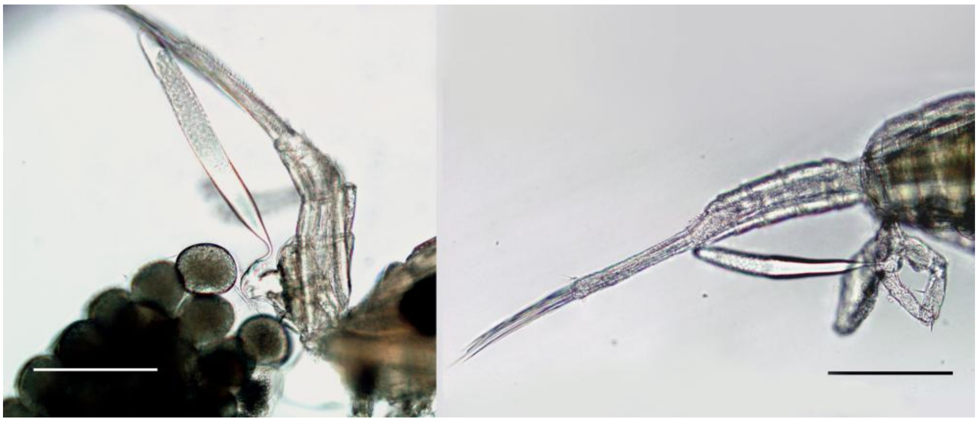

Figure 10. Right: photo of the spermatophore held in the tip of the left fifth leg of *E. affinis* male before placement in the gonopore of the female. Left: photo of the spermatophore attached to the genital urosome segment close to the egg sac. Horizontal bars are the 200µm scale.

Figure 11. Photo of three clearly attached spermatophores to the genital segment of the female's urosome of *E. affinis*. The tubular extensions of the spermatophores necks are attached to the same genital area and they are wrapped by secreted sticky cement.

Chapter IV: Particular physiological examples in *Eurytemora affinis*

Part 1: Occurence of intersexuality in a laboratory culture of the copepod Eurytemora affinis from the Seine estuary (France)

Authors : Anissa Souissi[1,2,3], Sami Souissi[1,2,3,*], David Devreker[1,2,3] & Jiang-Shiou Hwang[4]

[1]Université Lille Nord de France, F-59000 Lille, France

[2]USTL, LOG, F-62930 Wimereux, France

[3]CNRS, UMR 8187, F-62930 Wimereux, France

* Corresponding author: Sami.Souissi@univ-lille1.fr

[4]Institute of Marine Biology, National Taiwan Ocean University, Keelung, Taiwan 202, ROC

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Abstract

We show in this study that intersexuality can occur in the estuarine copepod *Eurytemora affinis* and we reported the presence of three intersexual copepod individuals from a laboratory culture of this species from the Seine estuary conducted at low temperature (7°C). These individuals presented both female and male characteristics. The prosome size and antennules of intersex individuals were similar to those of normal females, but all the other morphological details were more similar to normal males.

The appearance of the three intersex individuals in the culture at low temperature coincided with a decrease of food quality due to a feeding with *Rhodomonas marina* at its stationary phase. This induced a significant decrease in the mean clutch size and skewed sex-ratio in favor of males. The reduction of food quality in addition to low temperature of 7° C (which induced slow development) is suspected to be responsible of the appearance of intersex individuals. This stressful situation seems to propagate to the following two generations at low temperature in contrast to the case of the experiment at higher temperature 20°C where no intersex individual was observed. These results confirmed the role of food quality in sex determination of copepods. Moreover the role of early developmental stages and also the consequences of stressful conditions throughout different generations were discussed.

Introduction

Generally, sex determination in animals is controlled genetically by a chromosomal mechanism or epigenetically under the influence of environmental factors such as nutrition, temperature, pressure, population density, and parasitism (Fleminger 1985). In crustaceans, the sex determination is controlled by genetic factors but can also be influenced by environmental factors such as photoperiod and temperature (Sillett and Stemberger 1998). However, when the fitness (reproductive success) of an individual varies with its age or size, and when the equilibrium between males and females is compromised, sex change may be favoured (Allsop and West 2003; 2004). This is the “sex allocation theory” suggested by Charnov (1982). Animals can change sex during their lifetime using many strategies; they may be female first, male first, repetitive sex changers (Buston et al. 2004), or they may exhibit both male and female characteristics, as described by Atz (1964). Some authors used the term of gynandromorphism to define intersexuality (LeBlanc 2007; Olmstead and LeBlanc 2007).

In field populations, intersexual (gynandromorphic) individuals have been observed among various groups as crustaceans, molluscs, insects, spiders, ticks, frogs and fishes (Olmstead and LeBlanc 2007 and references therein). The occurrence of intersexuality in field population of crustaceans is a very rare phenomenon (less than 1%) and its causes and effects are unknown (Olmstead and LeBlanc 2007). Some studies showed that intersexuality is correlated to environmental factors, skewed sex ratio (Dunn et al. 1990; Dunn et al. 1996; Sillet and Stemberger 1998), or it can be a consequence of juvenile and adult parasitism (Swell 1951; Ianora et al. 1987). Ford et al. (2003) found that parasitism can alter the sexual development and induce a continued somatic growth and provoke intersexuality through incomplete feminization of male hosts. These authors report that intersex occurs only in old individuals.

Copepods are crustacean constituting a very important group among aquatic animals (Schminke 2007; Souissi et al. 2007). Their sexual dimorphism is expressed in pre-adult and adult stages by variations in size, modified antennules, asymmetrical fifth legs, numbers of urosomites, metasomal wings, caudal furca, genital complexes and tegumentary glands (Dharani and Altaff 2002). Intersexuality has been observed in copepod populations through modifications in their fifth pair of legs, antennules, genital shield and in other structures (Sillett and Stemberger 1998). The intersexuality in copepods is characterized by individuals showing the secondary sexual characteristics of the opposite sex while keeping their ability to reproduce as functional males or females (Sillett and Stemberger 1998). Most observed forms of intersexuality refer to masculinized females (exhibiting secondary male sexual characteristics) (Sillett and Stemberger 1998) whereas feminized males (exhibiting secondary female sexual characteristics) are rarely documented.

Recently Gusmão and McKinnon (2009) reviewed intersexuality and sex change in copepods and suggested that nutritional status (food quantity and quality) is the most important factor controlling sex determination in copepods. Moreover they considered that the difficulty of distinguishing intersexual individuals in copepods could explain the low number of publications reporting this phenomenon.

To our knowledge, most published studies on intersexuality have dealt with *in situ* observations of the natural copepod populations, and most reported results concerned few groups of calanoids (Gusmão and McKinnon 2009). The low number of publications of intersex occurrence in copepods makes it difficult to distinguish if some groups (or species) are more sensitive to sex reversal process. Moreover sex differentiation mechanism in copepods is still unknown and the presence of endocrine process is only extrapolated from studies carried on some crustacean groups (Gusmão and McKinnon 2009). For other groups such as fishes several examples of intersexuality were observed in heavily polluted estuaries and bays such as the Seine estuary and the Bay of Seine (Minier et al., 2000). Since few years a multidisciplinary research program targeting the copepod *Eurytemora affinis* was developed in the Seine estuary. Initially, the presence of morphological anomalies in earlier and late developmental stages (i.e. intersex) of *E. affinis* in the Seine estuary was strongly suspected. Excepting few observations of morphological anomalies in adults of *E. affinis* (Forget-Leray, unpub. data) the occurrence of intersex individuals from the field and also in the laboratory was not recorded (Devreker et al., 2008; 2009).

In this paper, we confirmed that intersexuality can occur in the estuarine copepod *Eurytemora affinis*. We report for the first time intersexuality in male individuals which were feminized, from a laboratory culture of the calanoid copepod *Eurytemora affinis*, a dominant member of the Seine estuary zooplankton community. Food quality and slow development at the lowest experimental temperature are responsible of these morphological aberrations. Moreover we consider the changes in three key life traits of the population (total production, sex-ratio, and fecundity of females) in the two generations before the appearance of intersex individuals, during the generation containing these anomalies, and two generations afterwards.

Materials and methods

Eurytemora affinis was first sampled in the Seine estuary in November 2006 and then cultured in the laboratory under controlled conditions. The culture was kept in 2 litre beakers of filtered sea water with salinity adjusted to 15, and maintained in a climate chamber at constant temperature. A continuous culture of *Rhodomonas marina* was also maintained in order to provide an adequate food for optimal development of *E. affinis* (Souissi, unpublished data). The cultures of *R. marina* were conducted in a climate room at 16°C and photoperiod of 12 h light: 12 h dark cycle. Under these experimental conditions the positive growth phase of *R. marina* was about two weeks. In order to avoid any food limitation the growth rate of the *R. marina* culture was calculated, it reached the highest value during the first week (0.35 d^{-1}). Therefore a linear relationship was also made between the cell counting (Y: 10^3 $cells.ml^{-1}$) and the fluorescence of *R. marina* measured by a spectrophotometer at the wavelength of 440 nm ($Y=3343.8*X-206.03$; $R^2=0.944$) allowed us to easily monitor algal culture without cell counting and also to avoid any food shortage during long-term experiments.

Copepod cultures were fed ad lib once a day with *R. marina* used during its exponential phase of growth (algal concentration: ~0.7-1 million $cells.ml^{-1}$). A small volume (~10 ml) of algae was taken then gently centrifuged in order to remove the culture medium without damaging the cells. Then the culture medium was replaced by filtered sea water adjusted to salinity 15. Finally food tubes were transferred to the climate room containing the copepod culture. After acclimation to the experimental temperature, each batch culture (2 L beaker) received non-limiting food algae giving a final concentration varying between 3900 and 5500 $cells.ml^{-1}$. This protocol was developed recently to avoid any excess of food given to *E. affinis* that may accumulate in the bottom of the beaker and could alter the feeding habits of *E. affinis*.

The copepods sampled in the low salinity zone of the Seine estuary during autumn (November, 20, 2006) were transferred to the Marine Station of Wimereux (France) to start the culture. First copepods were sorted in an aquarium filled with sea-water (a sterilized mixture of seawater filtered through 7 µm and deionised water) at salinity 15 and temperature of 10 °C. Copepods were fed with *Rhodomonas marina* and water was changed frequently in order to keep the culture under optimal conditions. This culture was maintained continuously during 3-months at 10°C then temperature was decreased to 7°C. When most adult females became ovigerous, we started a new culture with the new generation, acclimatizing the copepods to a temperature of 7°C during a total period of two months. After that, the experiment started by observing 6 successive generations over 11 months. Each new generation *n* was started by isolating 40 ovigerous females from the previous generation (*n*-1). The number of 40 females was chosen after preliminary trials of different initial conditions, and this number allowed the development of a cohort without excessive competition for food due to a high density (density-dependent effect). A lower number of ovigerous females can increase the demographic noise and affect the results obtained at the population level (Souissi et al. 2004). The remaining individuals of the previous generation (*n*-1) were fixed using buffered formalin for further measurements. For each generation *n* we examined a number of life cycle traits: the density (total number of individuals produced by the generation, N_n); the sex-ratio of adults (female: male), and the clutch size (Eggs sac^{-1} female^{-1}).

At the experimental temperature of 7°C the complete development of a cohort needs almost 6 weeks. Approximately, every 10-12 days on average copepods were concentrated using a small sieve of 33 µm mesh size and gently cleaned and then transferred to a new beaker with freshly prepared sea filtered water adjusted to salinity 15. The algal cultures were maintained during the complete experimental period at different phases of growth to guarantee for copepod culture a daily supply in *R. Marina* under its exponential phase of growth. One time, in the middle of the experiment, the algal cultures reached rapidly the stationary phase of growth and new cultures were inoculated. During one week copepod cultures were fed with the old *R. marina* cultures (stationary phase of growth) until the new inoculated culture reached the exponential phase of growth. Then the protocol continued as before until the end of the experiment. These problems of algal cultures were observed during the development of the fourth generation of *E. affinis*.

Adult males and females produced in each generation were carefully observed under a dissecting microscope. We observed anomalies in morphology of adults in the fourth generation. As a consequence, we identified three different categories of adults *E. affinis*: normal males, normal females, and three intersex individuals (feminized males). These intersex copepods had male fifth legs but right antennules without geniculation. In order to compare their morphology, the size of the prosome and the urosome were measured for all copepod individuals identified under an inverted microscope (OLYMPUS IX71). In addition, for males and intersex individuals, the sizes of the 15th segment of the right antennules and the basipodite of the left fifth leg were also measured. All the measurements were made using the image analysis software package Image J 1.41 (Rasband, 1997-2008).

Because of the low number of intersexual individuals observed during the study, it is difficult to use standard statistical tests to compare morphological characteristics of intersex and normal adults (i.e. males or females). As a consequence, we generated for each morphological character observed in normal adults an empirical probability density function (PDF) by resampling the observed values 10000 times. This procedure called 'bootstrap' allowed us to directly compare the value of each morphological character of intersexual individuals to the empirical PDF. For a given threshold value α (i.e. α=0.01) the statistical decision can be directly made by comparing the observed value ($Y_{intersex}$) to the cumulative probability distribution of 10000 mean values generated by bootstrap. For a unilateral statistical test, $Y_{intersex}$ is significantly different from the observed values in a normal adult when the probability of observing $Y_{intersex}$ is less than α or greater than ($1-\alpha$). This can be easily visualised with a graphical representation of the cumulative distribution of the generated PDFs. These analyses were done using the Matlab Software package (Mathworks, Inc.).

Results

Initial overall observations of all individuals revealed that intersex copepod individuals are more similar to the normal males than normal females (Fig. 1). Further observations of secondary sexual characters confirmed significant differences between intersex individuals and normal individuals of both sexes. The size of the prosome of intersex individuals is similar to that of females. The mean prosome length of the intersex individuals was 1034.99 µm, whereas it was 1003.38 µm for normal females and 954.25 µm for normal males (Fig. 2a). The same pattern was observed for the prosome width, it was 381.23 µm for intersex copepods and 363.96 µm for normal females, but for normal males it was 325.71 µm (Fig. 2b). Differences were also observed for the urosome measurements (Fig. 1). The urosome length was much smaller in the intersex individuals (307.37 µm) compared to the length of both normal males (415.13 µm) and normal females (363.96 µm) (Fig. 3a). On contrary, the urosome width for the intersex (101.79) was found to be more similar to that of normal males (107.00 µm) compared to normal females (152.21 µm) (Fig. 3b).

The main observable difference between intersex and normal individuals in our study is the shape of the antennules. Usually, the right side antennule of normal males of E. affinis is geniculated with 22 segments (fig. 4b) and normal females have two similar antennules on both sides of their body with 25 segments (fig. 4a). Intersex individuals showed antennules similar to the normal female ones with 25 segments and without any geniculation (Fig. 4c). The 15th segment of the right antennule constitutes the largest segment before the hinge for the male antennule (fig 4). The measure of this segment for the three types of adults revealed that the intersex have a 15th segment like the normal female's; in normal males this segment was 72.17 µm long and 75.85 µm wide but for the intersex individuals the length was 59.29 µm and the width was 27 µm (Fig. 5a).

The fifth pair of legs is one of the most important secondary sexual characteristic distinguishing differences between normal males and females in the majority of copepod species. Copepod females have two symmetrical fifth legs, whereas the fifth legs of males are asymmetrical. The observation of the fifth pair legs of intersex individuals showed strong similarities with normal male fifth legs, but there were some differences (Fig. 6). We measure the basipodite of the left fifth leg, which constitutes the most prominent part of those legs. The mean length of the basipodite of the left 5th leg was larger for normal males (175.63 µm) than for intersex individuals (140.74 µm), however the mean width of the basipodite was larger for intersex individuals (87.57 µm) than for normal males (71.81 µm) (Fig. 5b).

The statistical tests using bootstrap techniques showed that all morphological traits measured for the intersex individuals are significantly different from those measured for normal males and normal females with a highly conservative p-value ($p<0.0001$).

In general, the prosome size and antennules of intersex individuals are similar to those of normal females, but all the other morphological details are more similar to normal males. Consequently we suggest that these intersex individuals are feminized males.

All feminized males were observed at the 4th generation (F4), and figure 7 shows the differences of three life cycle traits (total number of individuals produced per generation, sex-ratio and mean clutch size) between F4, the two previous generations (F2 and F3) and the two successive generations (F5 and F6). The total number of individuals produced during F4 (591 individuals) was ranged between the values obtained at F3 (487 individuals) and those produced at F2 (692 individuals), but almost doubled during the last generations F5 and F6 (Fig. 7a). The sex-ratio was always unbalanced in favour of males with the highest values obtained during the generations F2 and F3 with the values 0.77 and 0.76 respectively (Fig. 7b). The sex-ratio during F4 was equal to 0.57 whereas the individuals produced during F5 and F6 were in majority males (sex-ratios 0.34 and 0.37 respectively). The mean clutch size of females during F4 was much lower than the values from the previous generations, and remained lower during the last two generations (Fig. 7c). The use of two-sample T-test confirmed that the mean clutch size of F6 was significantly different from all mean values of clutch size of the other generations F2 to F5 ($p<0.001$). On the other hand the mean clutch size was not significantly different between F2 and F3 and also between F4 and F5, whereas the difference between each generation from each group was highly significant ($p<0.001$). The stability of mean clutch size during the generations F4 and F5 and the significant increase of total individuals observed during F5 is explained by the higher survival of individuals compared to F4. The increase of survival may be explained by the improvement of food quality compared to F4, all other environmental factors staying constant.

Discussion and Conclusion

In the Seine estuary the copepod *E. affinis* has been suggested to be a good candidate to develop a new indicator of water quality because of its dominance of zooplankton community in the low salinity zone of many estuaries in the North hemisphere (Devreker et al., 2009). However during previous studies in the field (Cailleaud et al., 2007; Devreker 2007; Devreker et al., 2008) and also in the laboratory (Devreker et al., 2004; Devreker et al., 2009) we did not observe any intersex individual among *E. affinis* population from the Seine estuary. Moreover the occurrence of intersexuality in *E. affinis* was not reported in the literature.

In this paper we observed three intersexual copepod individuals from a laboratory culture of *E. affinis* from the Seine estuary conducted at low temperature (7°C). These individuals presented both female and male characteristics. The prosome size and antennules of intersex individuals were similar to those of normal females, but all the other morphological details were more similar to normal males. The morphology of normal adult males of *E. affinis* are characterised by prominent modifications expressed in the antennules and the fifth legs. The antennules are primitively geniculated on the right side only and the male uses it to capture the female's urosome before transfer and attachment of the spermatophore using the modified fifth pair of legs (Katona 1973; Sillett and Stemberger 1998; Ohtsuka and Huys 2001). The male is also characterized by a five segmented urosome. In contrast, the normal adult female has a three segmented urosome with an enlarged first segment, and a wing-like expansions of the posterior prosomal end margin that may provide a hydromechanical cue to the seeking male (Ohtsuka and Huys 2001).

Some authors suggested that intersexuality phenomenon can be caused by an unbalanced sex-ratio in favor of males. In the absence of females, some males can facultatively change sex to become functional females (Dharani and Altaff 2002). It may be also a response to poor environmental conditions for copepod health (Fleminger 1985; Sillett and Semberger 1998). It seems that in Calanoid copepods, genetic males can change to functional females (Dharani and Altaff 2002). More recently Gusmão and McKinnon (2009) proposed a reinterpretation of the size advantage model (Charnov 1982) suggesting that copepods can only change sex during the pre-adult stage. Moreover due to the absence of any direct evidence of the mechanism of sex determination in copepods, Gusmão and McKinnon (2009) used their own experimental observations on the calanoid copepod *Acrocalanus gracilis* to suggest that the sex determination in copepods is principally controlled by their nutritional status. In addition they considered that the possible point of sex change in copepods is the C4 stage that could be the last point in development in which both sexes are physiologically similar.

In our study we followed simultaneously the development of *E. affinis* from the Seine estuary during several generations at their optimal salinity 15 psu (Devreker et al., 2007) but at one cold temperature (7 °C) and one hot temperature (20 °C). The feeding of copepods with *R. marina* culture at stationary phase during one week negatively affected mean clutch size of *E. affinis* at both experimental temperatures. At 20 °C, *E. affinis* females developed after two weeks (regular development time) produced almost half mean clutch size (20.70 eggs.female^{-1}) compared to the mean clutch size of the previous six generations (39.20 eggs.female^{-1}) and also the following one (40.33 eggs.female^{-1}). At 7°C, the 4th generation coincided with the decrease of food quality and the appearance of the three intersex individuals. By considering the general law of temperature effect on the development of *E. affinis* (Dur et al., 2009) we estimated that only early copepodite stages C1-C3 faced the poor food quality. The mean clutch size of females in the cohort with intersexual individuals was significantly lower than the mean clutch size produced in the previous cohorts (F2 and F3). The decrease in fecundity can be explained by a decrease in algal quality (Hartwell et al. 1993; Koski et al. 1999). In fact, both experiments realized at 7 °C and 20 °C faced the same food quality conditions but no intersex copepods were observed at 20°C. We suspect that both low temperature of 7° C (which induced slow development) and a reduction of food quality due to the use of the algae in its stationary growth phase are responsible of these anomalies. Recently Berenike et al. (2009) showed that the biochemical composition of the diatom *Thalassiosira weissflogii* changed significantly from the exponential phase of growth to stationary phase and also during the senescent phase. These changes reduced to the half the egg production rate of the calanoid copepod *Acartia tonsa* in comparison with those fed with diatoms at exponential phase of growth. We can consequently suggest that a similar mechanism of changing biochemical composition in *R. marina* happened in our experiment when *E. affinis* was fed with the algae at its stationary phase.

Gusmão and McKinnon (2009) proposed that the main cue to change sex is food quality and quantity and that the sex change is more promoted in slow growing animals which face for longer low food quality than fast growing animals. This hypothesis could explain the absence of intersex individuals in our copepod culture at 20 °C. Moreover we can postulate that early copepodite stages (i.e. C1-C3) when they experience low food conditions could enhance sex change in copepods since C4 stage in *E. affinis*. Other studies dealing with the same copepod species already shown that either low food quality (Devreker et al., 2007) or the presence of endocrine-disrupting chemicals (Forget-Leray et al., 2005) could decrease the development rate of N6 and C1 stages. Therefore the metamorphosis from nauplii to copepodites could be a critical phase in the life cycle of copepods and should be considered in future studies of sex-determination in copepods.

In our study, the appearance of intersexual individuals in F4 at low temperature was accompanied by a decrease of the sex-ratio (F/M) that dropped from 0.77 and 0.76 (F2 and F3 respectively) to 0.57. The sex-ratio decrease continued in the subsequent generations F5 and F6 (0.34 and 037 respectively). In contrast at 20 °C the sex-ratio dropped only during the generation fed with the algae at stationary phase reaching 0.468. In fact, the mean sex-ratio was 0.796 in the previous 6 successive generations and rose to 0.790 in the following one. We can conclude that the combined effect of low temperature and poor food quality encountered during a critical phase of the life cycle of *E. affinis* skewed the sex-ratio in favor of males. This stressful situation seems to be propagated to the following two generations at low temperature in contrast to the case of the experiment at 20°C. In the Seine estuary (where our culture come from), Devreker et al. (2008) showed that the sex ratio (adult females: adult males) favors males with an average value of 0.65. This could be due to lower environmental quality (West et al. 2002) and water pollution (Cailleaud et al. 2009), or due to adaptation to the highly dynamic water column (Devreker et al. 2008). In the laboratory, the sex-ratio was variable depending on salinity and temperature (Devreker et al. 2007). At the lowest temperature (10°C), Devreker et al. (2007) obtained a sex ratio of 0.8 at salinity 15 and a sex ratio of 0.65 at salinity 5. Even if these values are close to those observed in the first two generations considered in our study, it is worth noting that the experimental protocols are different between both studies, and also the temperature used in our study was colder. Even at high temperature, when food conditions are optimal, the sex-ratio of *E. affinis* remained in favor of males as in the case of our experiment at 20 °C. We also observed in different experimental conditions that the effect of any additional stress (i.e. salinity) in *E. affinis* cultures favored the production of males (Souissi, unpub. data). In response to local conditions, some individuals adjust facultatively their offspring sex ratio in the proportion of males (Trivers and Willard 1973; Allsop and West 2004). The population sex ratio may depend on the details of male and female life histories and their clutch size (West and Sheldon 2002).

According to Charnov (1982), intersex individuals are larger than normal individuals of their respective sexes. This was the case of the intersex individuals observed in our study; they were significantly larger than the normal males. The larger size of intersex individuals may have been a consequence of endocrine dysfunction, which provoked delayed maturation manifested by continued somatic growth (Ford et al. 2004).

Intersexuality can reduce fecundity, lower embryo survival, delay maturation, and lower mating performance (Ford et al. 2004). Intersex individuals are expected to have reduced fitness compared to normal individuals because of the change of their behavior and their reduced reproductive output (Dunn et al. 1990). Even if intersexuality affects fecundity, its impact on population seems to be weak. Accordingly, intersex individuals that have all these limitations do not contribute greatly to the population dynamics. However, their presence in the population could be an indicator of a decline of environmental quality (Olmstead and LeBlanc 2007), as suggested by our study.

According to the low percentage of intersexuality in the field (less than 1%) (Olmstead and LeBlanc 2007) and the absence of dedicated protocols to study this phenomenon either in the field or in the laboratory, a long term trend of these observations is weak (Bayly and Shiel 2008). Thus, we suggest that studies of morphological anomalies have to be carried on in the future with appropriate techniques and careful check of taxonomy and morphological anomalies. As Gusmao and McKinnon (2009) we recommend more dedicated studies on the mechanisms of sex determination and differentiation in copepods. Moreover for future experiments more attention should be given to the early copepod developmental stages and also to the consequences of experimental conditions (i.e. low food quality) through different generations.

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Figures

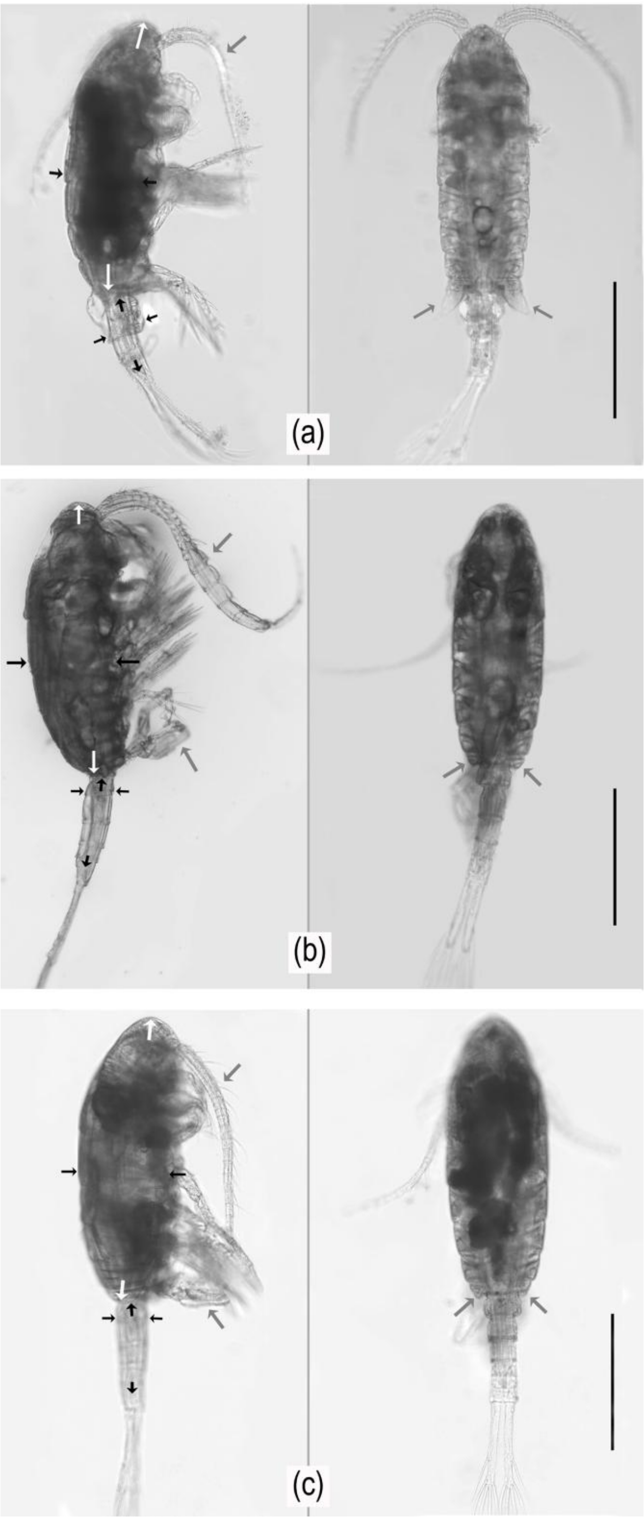

Figure 1 Lateral and dorsal views of a normal female (a), a normal male (b) and feminized male (c). **(a)** The main morphological characteristics are indicated with grey arrows: the non geniculated right antennule and the wings-like at the end of the prosome. The prosome length was measured from the top of the cephalosome to the end of the fifth segment of the prosome before the wings-like as shown by the white arrows. The prosome width was taken in the second segment of the prosome from the dorsal line to the line under and between the appendiges (see black arrows). The urosome length was measured over the whole three segments and the width was considered in the first segment which constitutes the largest one for a normal female (see black arrows). **(b)** Main characteristic morphology of male are indicated with grey arrows: geniculated right antennule , a developed fifth leg and a prosome end without wings-like. The prosome size measurements are indicated with arrows as shown in (a). The urosome length was measured over the whole five segments and the width were taken in the first segment (see black arrows). **(c)** The morphological characteristics of the feminized male are pointed with grey arrows: a non geniculated right antennule , a developed fifth leg and a prosome end without wings-like. The limit of measurements for the prosome and urosome sizes are indicates with arrows as for the normal male in (b). Scale bars = 500 µm.

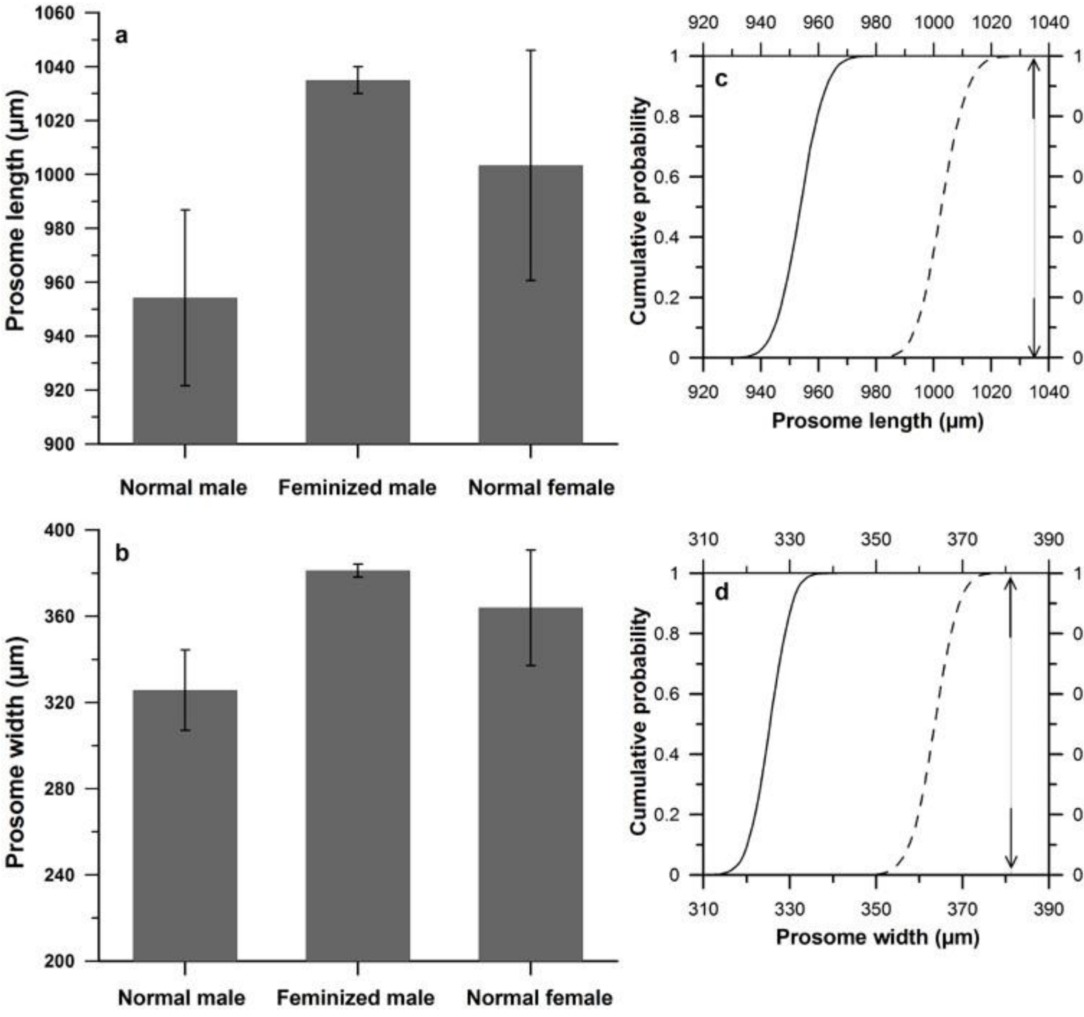

Figure 2 Prosome lengths (a) and widths (b) of both sexes of *Eurytemora affinis* adults (male and female) and the feminized male encountered in the 4th generation reared at 7°C. Vertical bars represent standard deviation. The cumulative probability distributions of prosome length (c) and prosome width (d) for males (continuous line) and females (discontinuous lines) were obtained by bootstrap resampling (10 000 times). Vertical thin line and both thick arrows indicate the observed value of prosome length (c) and prosome width (d) for the feminized individuals.

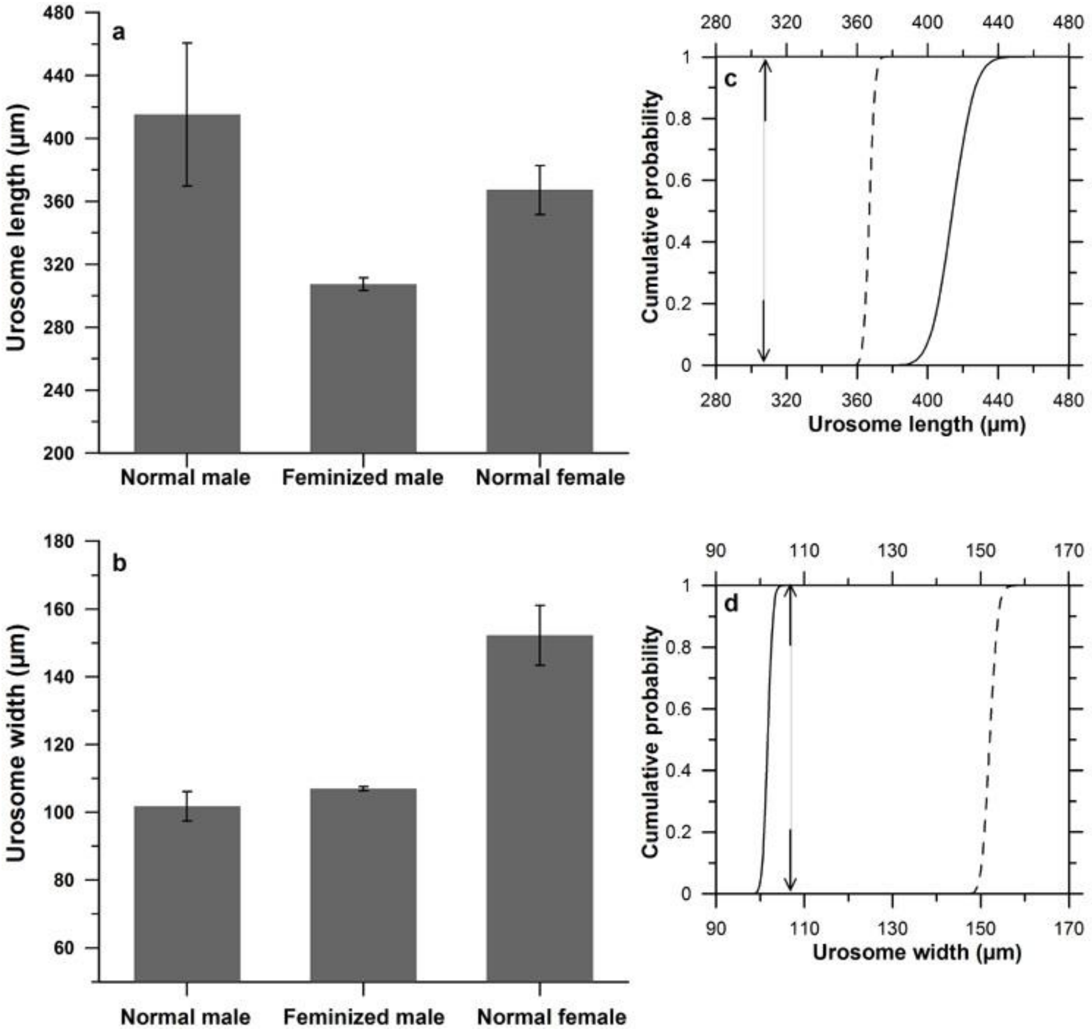

Figure 3 Urosome lengths (a) and widths (b) of both sexes of *Eurytemora affinis* adults (male and female) and the feminized males encountered in the 4th generation reared at 7°C. Vertical bars represent standard deviation. The cumulative probability distributions of urosome length (c) and urosome width (d) for males (continuous line) and females (discontinuous lines) were obtained by bootstrap resampling (10 000 times). Vertical thin line and both thick arrows indicate the observed value of urosome length (c) and urosome width (d) for the feminized individuals.

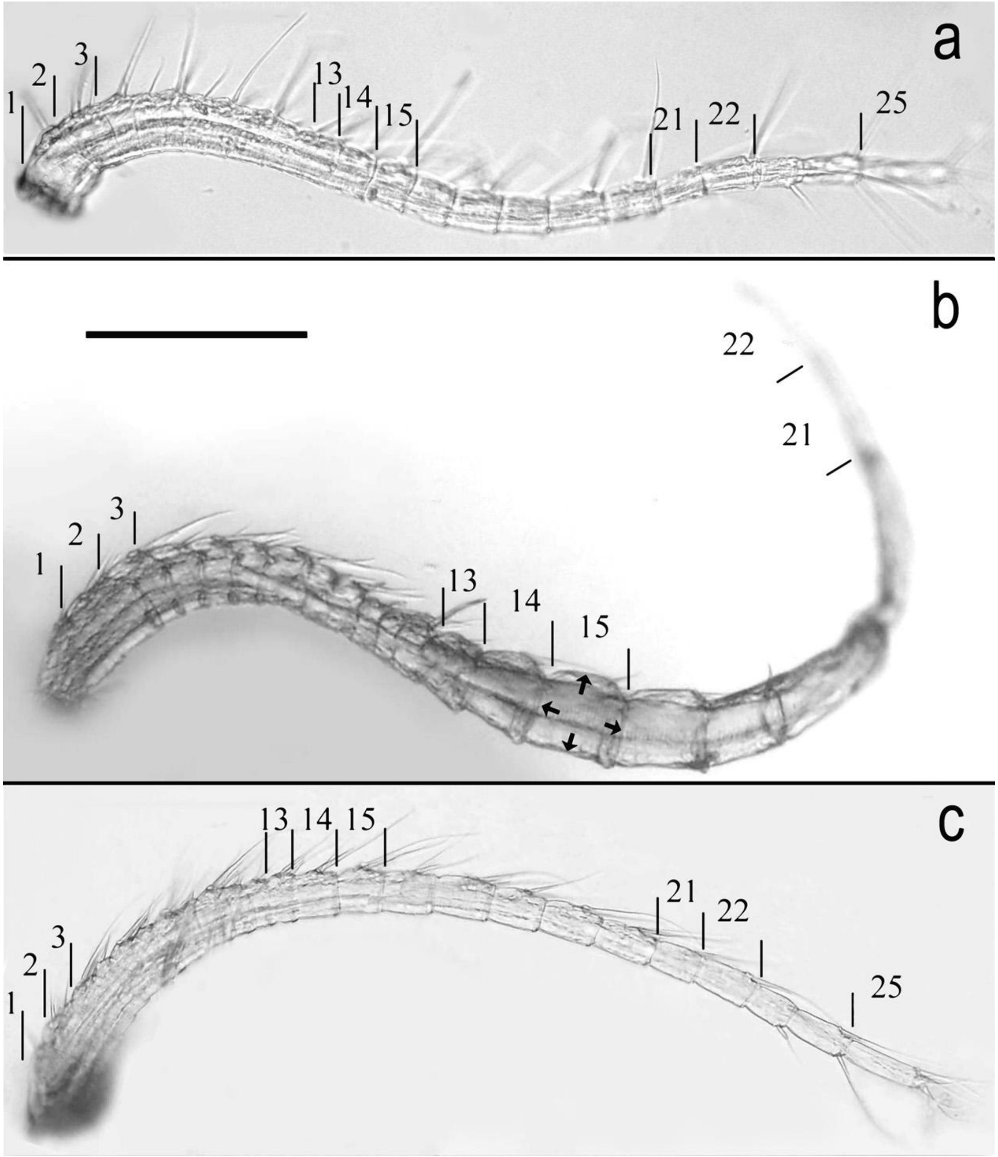

Figure 4 Different types observed of antennule: of normal female (a), of normal male (b) and of feminized male (c). Numbers show the first three segments (1-3), segments 13-15 and segments 21-22. The last segment (25) in normal female (a) and feminized male (c) is also labelled. The measurements of the 15th segment in the geniculated antennules (b) are shown by arrows. Scale bar = 200 µm.

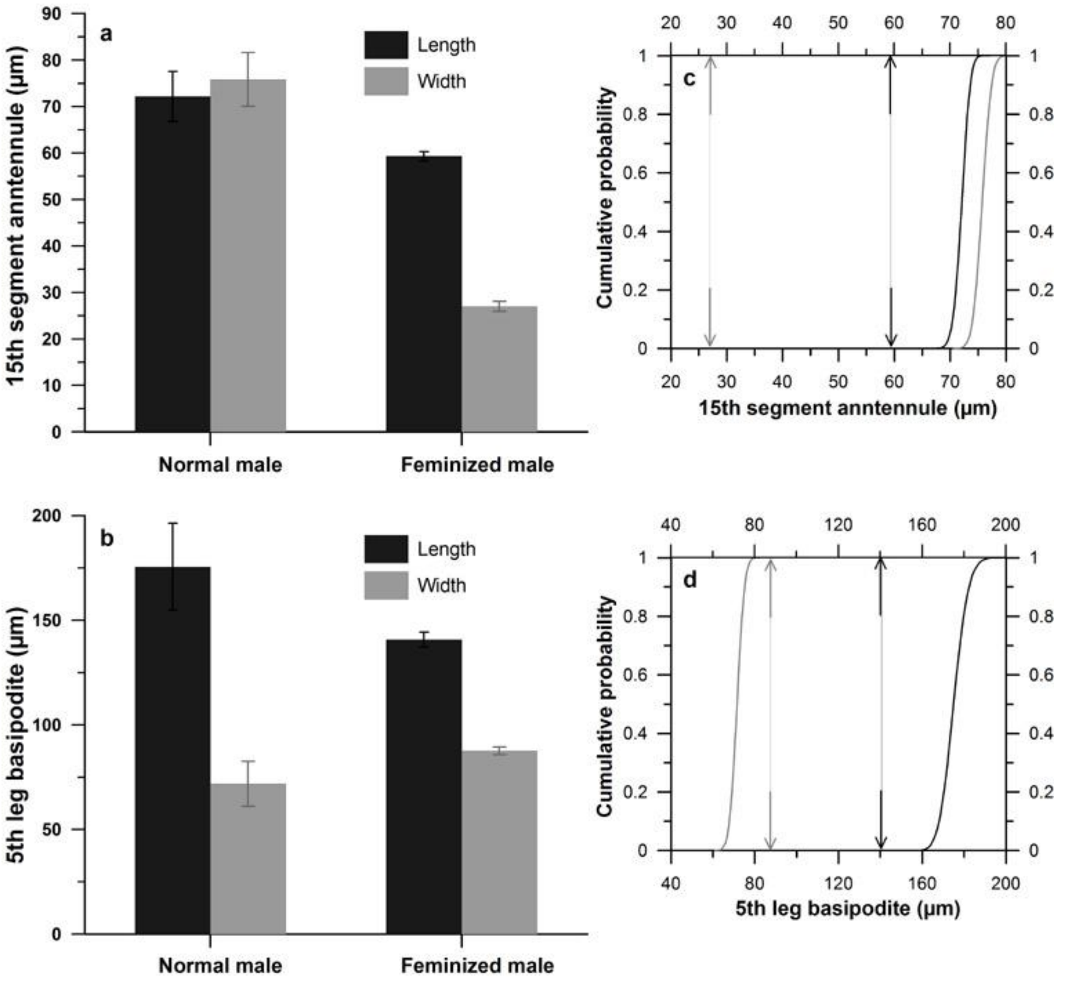

Figure 5 Mean sizes of the 15th segment of the right antennule (a) and the 5th leg basipodite (b) of a normal male and a feminized male of *Eurytemora affinis*. Vertical bars represent standard deviation. The cumulative probability distributions of the 5th segment antennule's length (black curve) and width (gray curve) (c) and the 5th leg basipodite's length (black curve) and width (gray curve) (d) for normal males were obtained by bootstrap resampling (10 000 times). Vertical thin line and both thick arrows indicate the observed value of the length (black) and width (gary) of each secondary sexual character observed in feminized individuals.

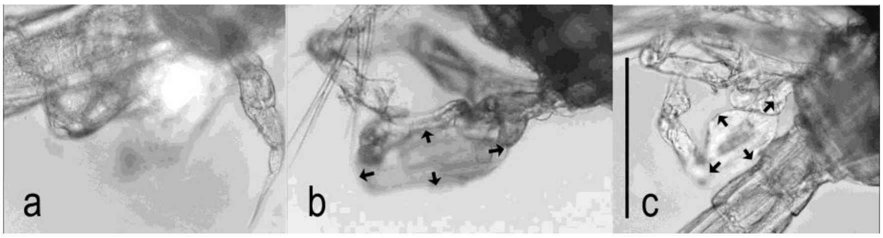

Figure 6 Different types observed of fifth legs: of normal female (a), of normal male (b) and of feminized male (c). The sizes of the basipodite of the left 5th leg were taken as shown with arrows (in b-c). Scale bar = 100 µm.

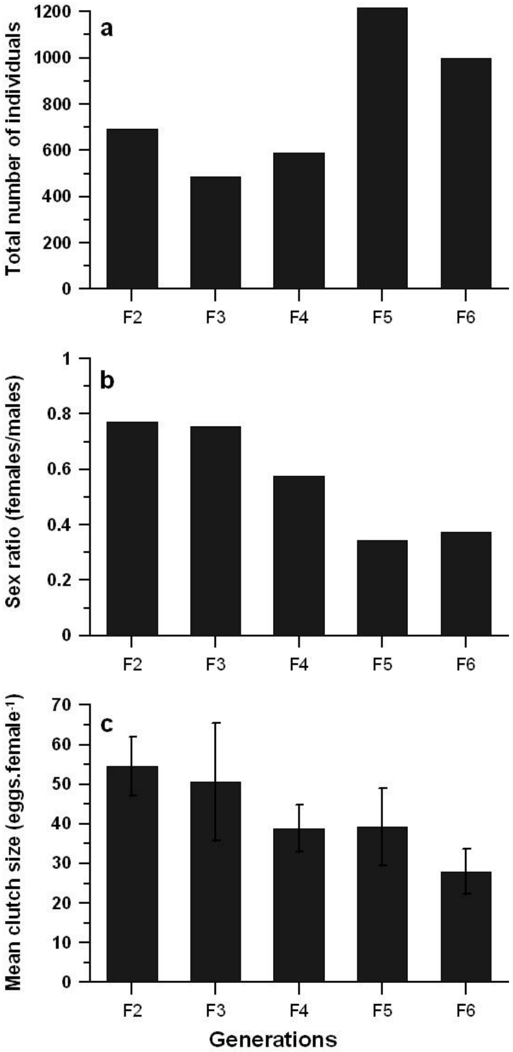

Figure 7 Total number of individuals produced per generation (a), sex-ratio (adult females: adult males) (b) and clutch size (c) of *Eurytemora affinis* cultured during 6 generations at 7°C. The feminized males are observed at the 4th generation. The detailed information about the different life traits is obtained for the generations F2 to F6. Vertical bars represent standard deviation.

Part 2: Effect of peritrich ciliates as epibiont on the behavior and the mating success of the copepod Eurytemora affinis

Authors : Anissa SOUISSI[1,2,3], Sami SOUISSI[1,2,3], Jiang Shiou HWANG[4]

[1]Université Lille Nord de France, F-59000 Lille, France

[2]USTL, LOG, Station Marine de Wimereux, F-62930 Wimereux, France

[3]CNRS, UMR 8187, F-62930 Wimereux, France,

[4]Institute of Marine Biology, Zooplankton and Coral Reef Laboratory, National Taiwan Ocean University, 202 Keelung, Taiwan

Abstract

Epibiosis relationships in aquatic ecosystems usually involve planktonic microcrustaceans as hosts and a variety of micro-organisms like bacteria, algae and ciliate as epibionts. This relationship can affect the hosts in their feeding due to competition with the epibionts, induce decrease in their reproduction and increase susceptibility to predation because their swimming as well as escape movements are limited. In the literature, several studies already mentioned the presence of epibionts in many calanoid copepods. However, their consequences on behavior are scarcely studied. The difficulty of maintaining cultures of infested copepods could explain such lack of our understanding on potential interactions between epibionts and calanoid copepods as hosts.

We study here the swimming behavior of the estuarine copepod *Eurytemora affinis* before and after infection by its most common ciliate peritrich epibiont *Zoothamnium* sp., Copepods were collected from the low salinity zone in the Seine estuary (France) during late winter 2009 and then maintained in the laboratory. The behavior of infested *vs* healthy copepods was recorded by using 2D infrared techniques. The presence of epibiont seems to negatively affect the behavior of its host in terms of swimming activity (motion, velocity, orientation) and mating success. The presence of high number of attached epibionts reduced the fecundity and the survival of copepod females. The trajectories of copepods were analyzed by using a set of appropriate statistical methods.

Most of studies dealing with epibiosis between copepods and ciliates from estuarine ecosystems have been carried in the Chesapeake Bay in USA and also in Baltic Sea (brackish water). To our knowledge our work is the first one carried in the Seine estuary ecosystem.

Introduction

Epibiosis is basically a commensal relationship However, several studies showed that epibionts can have negative effects on some life cycle traits of the host; as a decrease in reproduction, growth and survivorship and an increase of the susceptibility to predation by limiting their swimming and escape capabilities (Visse 2007).

The epibiotic relationship has been observed in copepods by several authors (Turner et al 1979, Valbonesi and Guglielmo 1988, Chiavelli et al 1993, Green and Shiel 2000, Visse 2007, Utz and Coats 2005a, 2008; Utz et al 2008a). Utz and Coats (2005a) studied the epibiont of the estuarine calanoid copepod *Eurytemora affinis* from the Chesapeake Bay in USA, and they found that the common epibiont of *E. affinis* is the sessile peritrich ciliate *Zoothamnium intermedium* characterized by an asexual life cycle. The life cycle of this ciliate alternated between attached feeding stages named the trophont and a free swimming dispersal stage the telotroch. The formation of telotroch in colonial or solitary peritrishes results either from asexual reproduction or by direct transformation of the zooids in response to unfavorable environmental conditions.

In the field, epibionts are present in very low or undetectable numbers. Their prevalence is influenced by some factors like the fluctuation in phytoplankton and bacteria populations, influx of freshwater in the system, pollution and mainly the host abundance. These factors also control the colonization of the copepod species by epibionts. Most of the studies dealing with the relationship between epibiont ciliate and their host only focused on the effects on host survivorship and reproduction (Kankaala and Eloranta 1987, Xu and Burns 1991, Xu 1992, Weissman et al 1993, Utz 2008a). But no deep studies explored the effect of this close relationship on the behavior of the host and its mating success. In fact, in this work, we try to explore this pathway and analyze the possible effect of these epibiont ciliate on the swimming and mating behavior of the estuarine copepod *E. affinis.*

Materials and Methods

The copepods *E. affinis* were sampled in the Seine estuary at the low salinity zone, during late winter at the end of February 2009. These copepods were then transferred to the laboratory and maintained in batch culture under controlled conditions of optimal salinity 15 and a temperature comprised between 16 and 18°C and *ad libidum* supply of food (the red algae *Rodhomonas marina*). The observation of the animals, after the sampling, revealed the presence of some copepods with few epibiont ciliates on their body. So, we isolated one aquarium with clean animals and kept the other batch culture aquarium. The identification of these epibiont ciliates were checked and compared to those described by Utz and Coats (2005a) on epibionts of our common studied copepod species *E. affinis*

One month later, the aquarium containing initially some infected individuals showed a strong infestation on the whole body of all copepods, whereas, the other aquarium still containing clean copepods. From these aquariums, two sets of experiments were initiated to compare control conditions represented by the copepods from the aquarium without epibiont and the condition with infected copepods, in order to record the swimming behavior of each sex, then their mating. For that 10 females and 10 males were sorted carefully from each aquarium and transferred to small aquariums of 10 cm^3, in order to acclimatize them before starting the recording. After one hour acclimation, each small aquarium containing 10 females or males was transferred to the recording dark room and calibrated with the infra-red camera then kept in total dark for 30 minutes of recordings. After the individual sex record, males and females were mixed in each control and infected condition then acclimatized for 15 minutes (to stabilize the possible suspended matter in the aquarium) after that transferred to the recording room for one hour record at the same previous circumstances. At the end, from each condition, 3 records were obtained. One week later, the infected aquarium was checked again and 10 other females and 10 males were sorted to continue the same recording procedure. This experiment was also repeated the following week until we observed the remove of the epibionts from the body of the copepod. The number of copepods was not sufficient to make more experiments (high mortality).

The records obtained, from each condition and each date, were checked and prepared, using adobe premiere. Afterward, each record was calibrated to permit the manual track of individual trajectories image by image using TrackIt Software, in order to extract the swimming trajectory of each copepod. The data base obtained allowed the extraction of the trajectories and the selection of the adequate ones.

The records containing males and females, for each condition, were also carefully observed to count the number of successful and unsuccessful mating with registration of the duration of each of these events.

Results

According to the description of Utz and Coats (2005a) on the epibionts of *E affinis* in the Chesapeake Bay and after comparison to those infesting the copepods in our study, we deduced that it is the genus *Zoothamnium* and most likely the species *Zoothamnium intermedium*.

Analysis of male and female trajectories in control and infected conditions

The analysis of the control male trajectories revealed that the male explore a large volume with a mean speed of 2.40 ± 4.91 mm/s whereas, the infected male showed a trajectory characterized by a vertical behavior with many irregularities and showed a mean speed lower than the control male (1.71 ± 2.40 mm/s) (Fig. 1). The probability density function (PDF) of the instantaneous speed gave a linear pattern in log-log plot for control male and showed a difference in the slope and in the extreme values for infected males. These differences in the PDF mean that there was less jumps in infected males swimming behavior (Fig. 2).

The trajectory of the control female presented a mean speed lower than the male with 1.42 ± 2.76 mm/s but, in contrary, showed a globally similar trajectory and mean speed (1.38 ± 2.65 mm/s) compared to the infected female (Fig. 3) with in addition a similar PDF of instantaneous speed (Fig. 4).

Dynamic of the epibiotic relationship between E. affinis and Zoothamnium sp.

The weekly observation of the infection state of the copepods revealed that the proportion of infestation decreased in the second week showing cleaner individuals but with new formation of epibiont colonies (Fig. 5). The number of copepods decreased also compared to the first observation. In the following week, more healthy copepods were observed showing bodies covered by vestige of old and died colonies of epibionts whereas the number of both epibiont and hosts decreased dramatically in the aquarium (Fig. 5).

Effect of epibionts on the mating success of E. affinis

The highest effect of epibiont infestation on *E. affinis* was observed in the mating success. In fact, the control copepods showed 6 successful mating and 9 unsuccessful ones in one hour, whereas, at the same condition, the infected copepods succeeded only on mating a single time within one hour. After one week, the copepods failed to realize any mating. It is only after two weeks when the number of epibionts and also hosts decreased that copepods accomplished 5 successful mating without showing any unsuccessful one (Fig. 6). The differences were notable also for the duration of successful mating. Indeed, for control copepods, a successful mating lasted in average 1 minute and 28 second but after two weeks of infestation by epibiont, copepods spent almost double time to succeed a mating (2min04sec). During the strong infestation in the first week, the single mating made was very short of only 42 seconds.

Discussion

Most studies on epibiosis with copepods focused on the description of this relationship, among them some dealt with the effects of peritrich ciliates on some key life cycle traits (survival, reproduction...etc) of copepods. However, the effect of this relationship on the swimming behavior and mating success of the host was mostly suspected without direct quantification.

Utz (2008b) described the mechanisms that led to peritrich epibionts to select their host on the basis of space availability and the access to resources. Some epibionts can assess their host, by using chemicals, because they need a specific living substrate composed by a single species or a group of species. The life cycle of the epibiont involving motile stages is well adapted to the morphological changes as well as to life cycle constraints (i.e. growth, molting, etc.) of the host (Wahl 1989; Utz and Coats 2005b).

The epibiont *Zoothamnium intermedium* specific of the copepod *E. affinis*, can settle on all parts of the host body, as observed in our study (see photos in Fig. 5) and the earlier work of Utz (2003). The intensity of epibiont infestation can be variable in time and space and could engender almost a complete cover of the copepod body including antennules and appendages. This stage of infestation was reached in our culture in one month period, suggesting an exponential growth of the epibiont during this incubation interval resulting from favorable conditions (host availability and optimal medium conditions in the aquarium). The presence of a high density of epibionts will negatively affect the host directly through the limitations of its movements and indirectly by competing for food (Kankaala & Eloranta 1987) leading at the final stage to shorten the life span of their host (Visse 2007).

Because feeding and mating of copepods occur at small scales and often involving swimming behavior (Hwang et al. 1994), any mechanical or chemical disturbance of these processes can alter their realization. Consequently, we focused in this study on the effects of the epibiosis on these small scale processes. We therefore showed a clear difference in the PDF of swimming speed in males between control and infested individuals, whereas, no significant differences in speed were observed for females. We concluded that males of *E. affinis* known to be more active than females (Katona 1973; Michalec et al. 2010) were more sensitive to the epibiont infestation than females. The shape of the trajectories of males and their PDFs were similar to those observed under the effect of sub-lethal exposure to nonylphenols (Cailleaud et al. 2011). We can thus conclude that the vulnerability of males and females of *E. affinis* to epibiont is different. The higher resistance of females was already demonstrated in heat chock treatments (Bradley and Ketzner, 1982). This difference could be explained by the need of males to maintain a high swimming activity to seek for potential mates, whereas, females are less motile (Katona 1973; our personal observations) and could be more resistant in case of infestation. From physical point of view, the female evolved to carry a heavy egg sac (Chapter II) could be less affected by the additional weight imposed by epibiont colonies. But when the colonization reached the swimming appendages, both males and females suffer from this infestation. We observed that when epibiont density was very high, the sinking phase of swimming behaviour became frequent and the fast swimming phase (jumps) was very rare mainly for the male. The logical consequence of this behaviour is an alteration in both feeding and mating rates. In fact, during the first observation coinciding with the peak of epibiont infestation the mating behaviour was significantly reduced (or absent). Then, a high mortality of copepods was induced during the 2 following weeks, after the maximal infection rate. The high mortality of copepods (compared to the control aquarium) could reflect the critical physiological state of *E. affinis* cultured in presence of epibiont. The high mortality of reproductive stages, the significant decrease of copepod fecundity (absence of mating) and the drop of recruitment (nauplii) contributed to the collapse of the population in the infested aquarium. The high mortality of the host provoked a decrease in the number of attached epibionts. In fact, epibionts start to form telotroch after the death of the host within 7 hours

(Utz 2003; Utz and Coats 2005b). The telotroch formation in sessile free-living or epibiont peritrichs occurs as a result of binary fission, in order to find a new substrate for attachment (Nagasawa 1986). The dispersal and non-feeding stage represents a critical phase in the life cycle of epibionts because they need to find a suitable substrate for attachment in a relatively short period (Frenchel 1965, Clamps 1973; Utz and Coats 2008). In addition, the telotrochs of *Z. intermedium* performance decreases with age showing a maximum success of attachment to hosts, in the early instants of the first hour of telotrochs formation (Utz and Coats 2008). In the case of *Z. intermedium*, the high mortality of the host immediately initiated massively the dispersal stage in a limited aquarium volume (no possibility to seek for new hosts) and engendered the mortality of the epibiont as predicted by the cyclic dynamics of the epibiont-host abundances (Utz and Coasts 2008).

The dramatic drop of epibiont number (colonies and dispersal stages) led to the survival of the most resistant copepods that probably improved their immunity (Kurtz 2007). These copepods presented a very low infection (see Fig. 5), recovered their “normal” swimming behavior and improved their mating success, compared to the control conditions (see Fig. 6).

Our experimental observations confirmed that the exponential growth of the epibiont population requires a high abundance of its host. For this reason, their prevalence in the field often corresponded to the maximum abundance of the specific host (Hirche 1974, Chiavelli et al. 1993; Visse 2007). In the Seine estuary, this level of infestation recorded in the laboratory culture was never been recorded in the field. The number of attached epibionts on *E. affinis* in the field was always very low when observed in the samples, whereas, in the Chesapeake Bay situated in southern latitude compared to the Seine estuary, such observations can occur more often (Utz and Coats 2005a). This testifies that the environmental conditions in both estuaries are different and could affect the epibiosis relationship. Moreover, the isolated population of infested *E. affinis* was sampled by the end of winter which could be a critical phase in the development of *E. affinis* in the Seine estuary. In fact, this period is mainly composed by over-wintering old adult stages where the levels of bioaccumulation of pollutants are the highest (Cailleaud et al. 2007). These individuals could be physiologically weakened and more vulnerable to such infestation. After the critical phase, the new generation of *E. affinis* develop during late spring early summer reaching high densities (Devreker et al. 2008; 2010) did not showed a presence of epibiont (or at undetectable level). This confirms that the presence of epibiont could be considered as an indicator of the physiological status of the copepod as well as the water quality (Henebry and Ridgeway 1979; Utz and Coats 2005a).

We showed, in this study, that laboratory mass cultures can offer optimal conditions for the development of epibionts. Therefore, it is important to consider the presence of epibionts on newly sampled individuals prior to initiate copepod cultures.

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Figures

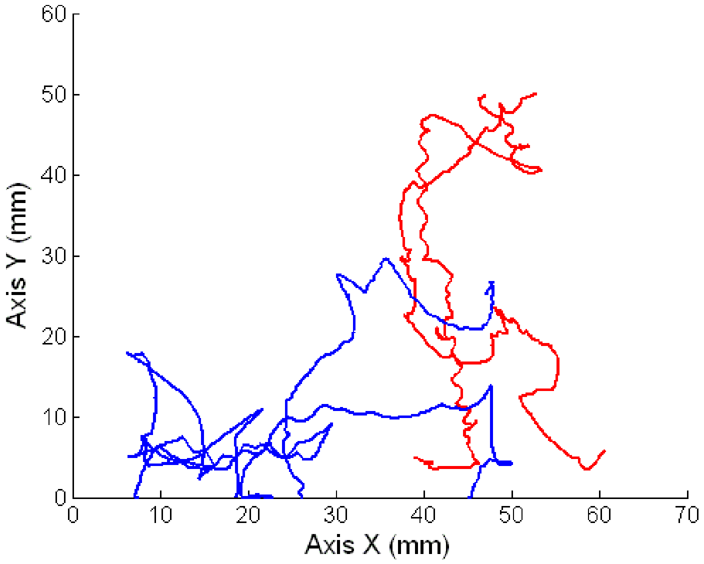


Figure 1. Examples of trajectories of *E. affinis* males in control (blue) and highly infested individuals (red).

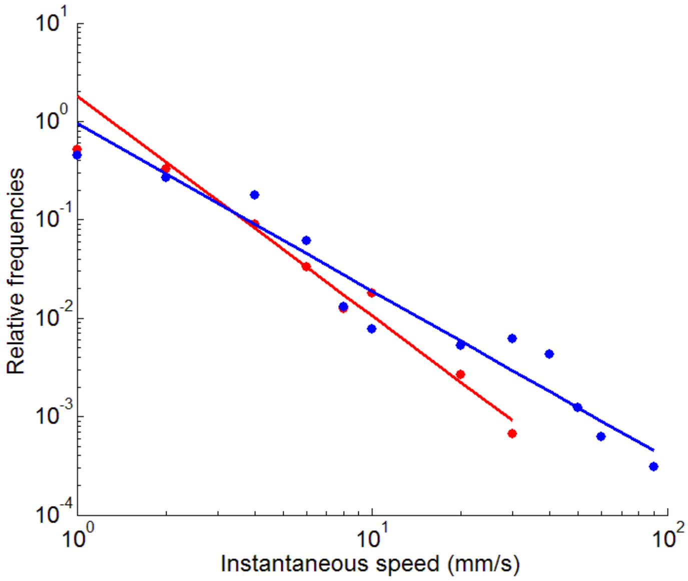


Figure 2. Observed (symbols) and fitted (continuous lines) probability density function (PDF) of the instantaneous speed of *E. affinis* males in control (blue) and infested (red) conditions. The PDFs are shown in log-log plot.

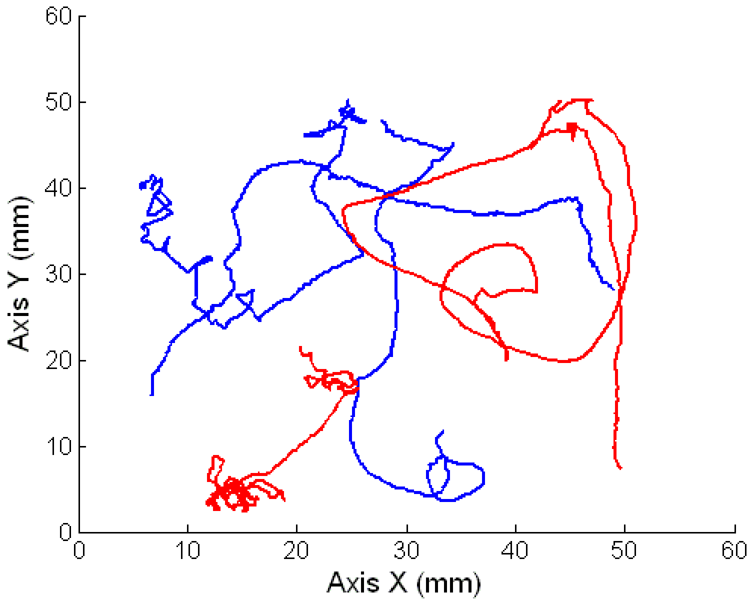


Figure 3. Examples of trajectories of *E. affinis* females in control (blue) and highly infested individuals (red).

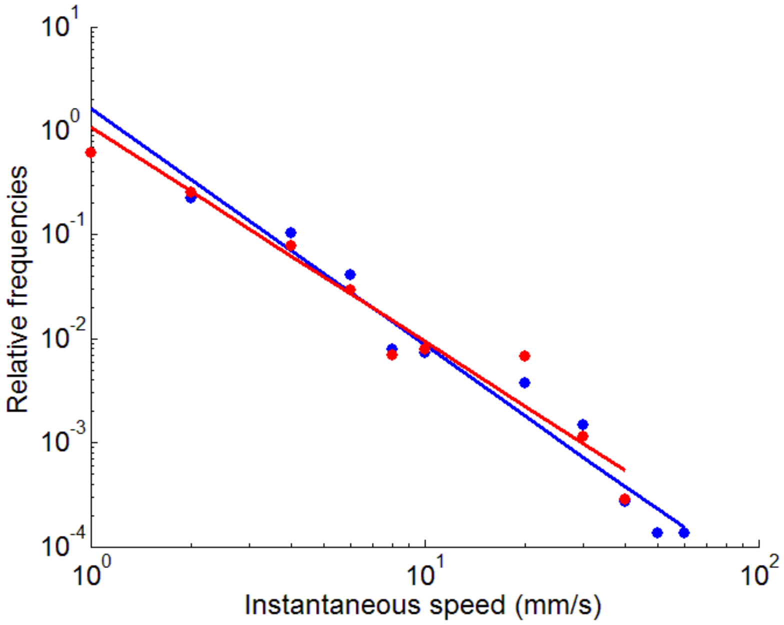


Figure 4. Observed (symbols) and fitted (continuous lines) probability density function (PDF) of the instantaneous speed of *E. affinis* females in control (blue) and infested (red) conditions. The PDFs are shown in log-log plot.

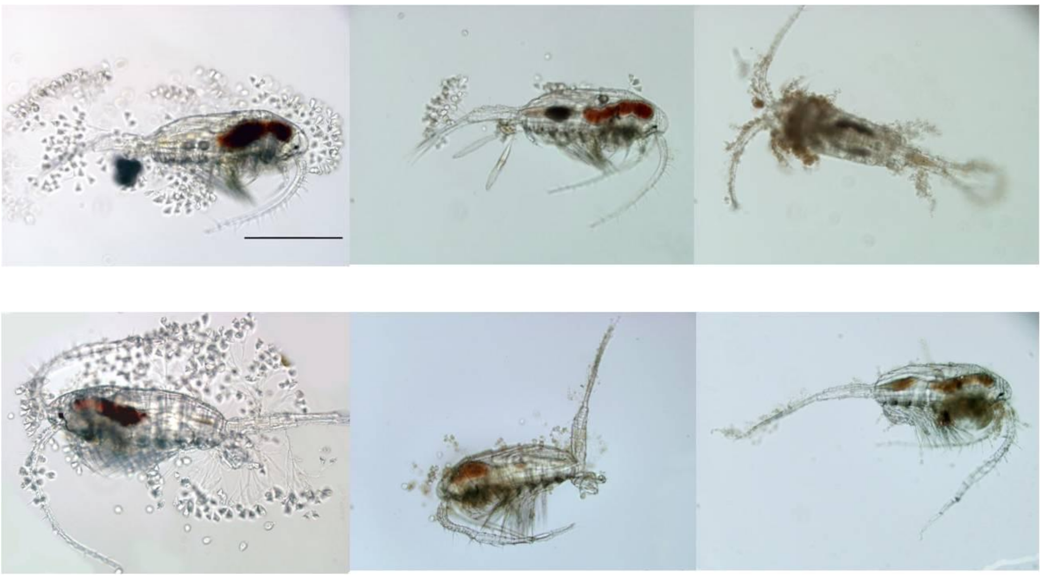


Figure 5. Series of photos showing the temporal evolution (from left to right) of the decreasing level of infestation of *E. affinis* females (photos on the top) and males (photos on the bottom). Horizontal bar is 500µm scale.

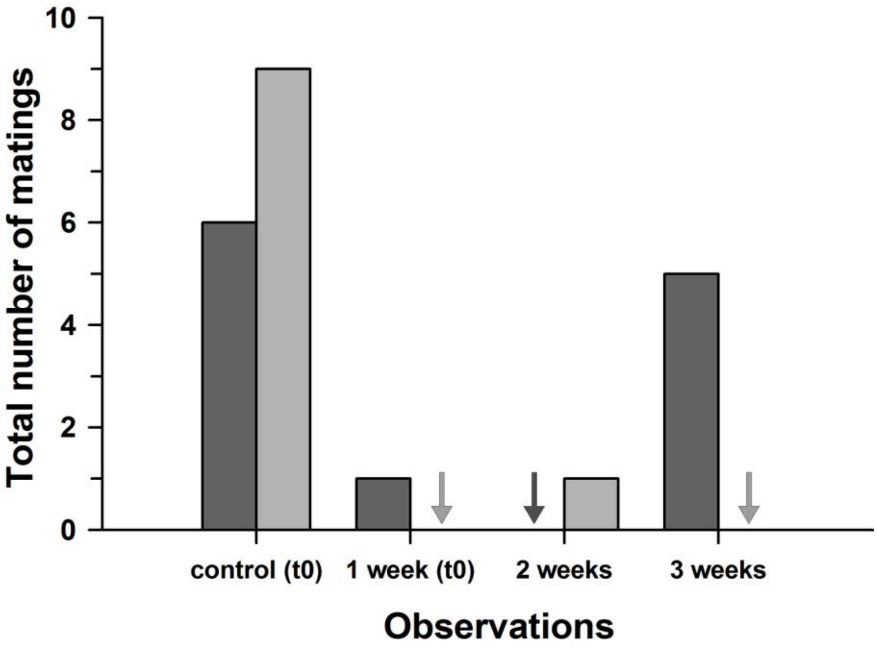
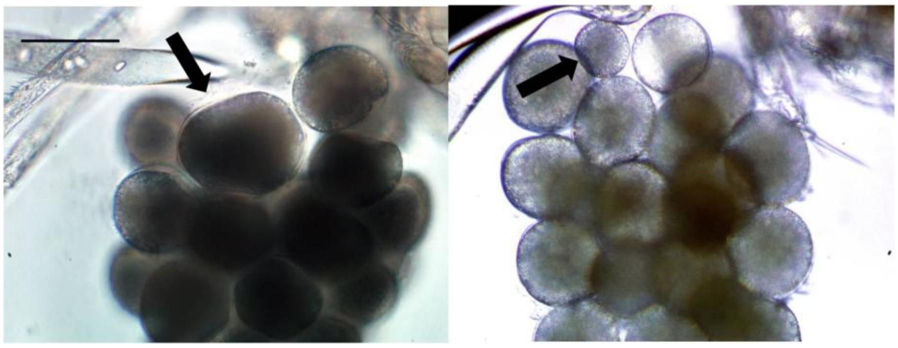


Figure 6. Total number of mating obtained after 1h of recording 10 males and 10 females, followed during three weeks and compared to control condition. Dark gray bars correspond to the number of successful mating and light gray ones represent unsuccessful mating. The black arrow designates the absence of successful mating records in the 2nd week. The gray arrows point the absence of unsuccessful mating records.

***Part 3*: Examples of morphological abnormalities in Eurytemora affinis**

1- Abnormalities in egg size



Opposed photos of occurrence of abnormal egg sizes in a single egg sac; with a very big egg in the left photo (originated from an ovigerous female from F17 at the culture condition T24S25) and a very small one in the right (originated from an ovigerous female from F15 at the culture condition T20S15). Arrows designate the eggs. Top left horizontal bar is the scale (100µm)

The presence of a single (sometime two) bigger egg in the sac compared to the other eggs from the same clutch occurred 10 times in the laboratory experiment of copepod culture from the Seine estuary in all conditions (one time in T20S15, 3 times in T24S15, two times in T7S15, one time in T20S25 and 3 times in T24S25) we noticed that this event occurred more frequently at temperature 24°C but is still rare event with only 10 observations among 8764 egg measures.

The presence of very small egg in an egg sac compared to the other of the same clutch of an ovigerous female was exceptional event happening only two times (once at T20S15 and once at T24S15) (observation frequency is 2/8764).

2- Abnormalities in egg shape

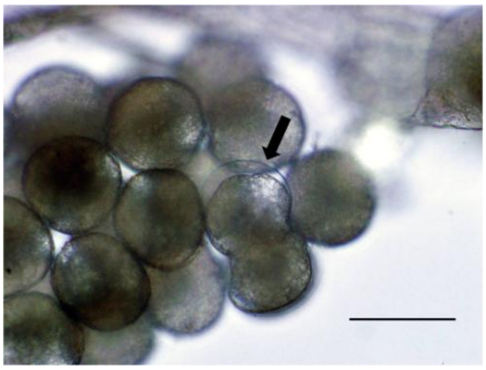


Photo showing the presence of a differently shaped egg in an egg sac (designated by the arrow). The horizontal bar is the 100µm scale

In the egg sac of one ovigerous female from the laboratory culture at the condition T20S25, an egg that seemed to be a fusion between two eggs was observed and occurred only one time among 8764 observations. We suspect that due to a strong egg packing constraint in the egg sac the pressure on the eggs induced the crack of two eggs that the contents merged.

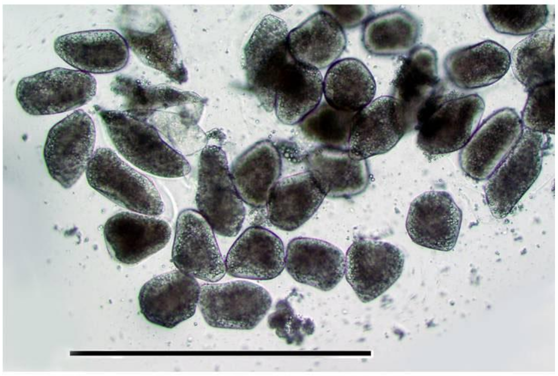
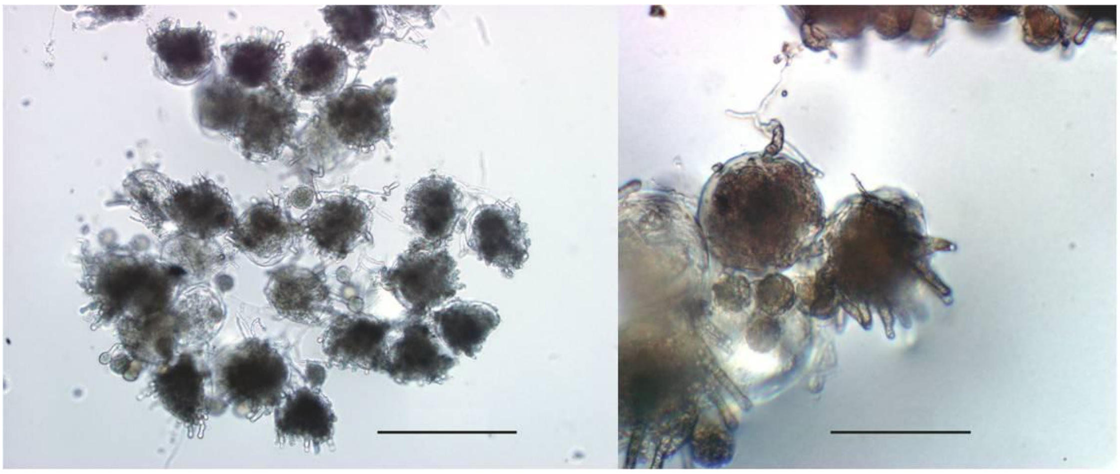


Illustration of an example of deformed eggs. The horizontal bar is the scale (500µm)

Deformed eggs were observed only in eggs of ovigerous females from the field mainly in one date sampling from the Seine estuary. Such event was observed 9 times among 18 ovigerous females in 10-02-2010. The same event was observed one time among 7 ovigerous females analysed from Montmagny (St Lawrence estuary) sample during may 2009. We suspect strongly that such eggs were not viable.

3- Abnormalities in the general state of the eggs and the egg sac



The left photo (scale = 200µm) shows a number of eggs from a single clutch infested by fungi. The right photo is a zoom on two infested eggs by fungi (scale = 100µm)

The occurrence of eggs infested by fungi was observed among eggs of ovigerous females sampled from St Jean Port Joli in St Lawrence estuary and was noted 8 times among 20 observations of ovigerous females.

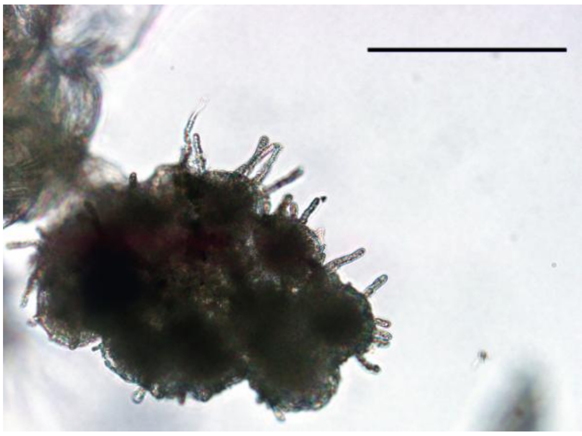


Photo of an egg sac infested by fungi. The horizontal bar is 200µm scale

Ovigerous females with an egg sac infested by fungi were registered 5 times among 11 observed individuals in a sample from Chesapeake Bay taken during April 2008.

4- Deformation in the body of *E. affinis* ovigerous females

Ovigerous female of *E. affinis* containing deformation on the urosome (left photo scale = 500µm). the right photo is the zoom of the rectangle part designated by the arrow enlarging the deformated part of the urosome (scale = 200µm)

Some observed ovigerous females presented a protuberance in the last segment of their urosome, we can confer this deformation to a tumor or other desease. These deformations were observed (5 times among 18 observations) in ovigerous females sampled from the seine estuary during february 2010.

Remark: We noteced that the same field sample of (10-02-2010) obtained from the Seine estuary contained ovigerous females with deformations in their urosome but also in the shape of their eggs.

General Conclusion & Perspectives

In this thesis, we developed a pilot study to explore the plasticity of the copepod *E. affinis* from different angles. We proposed a multigenerational protocol allowing to study the effect of acclimatation conditions and also to track maternal and trans-generational effects. The need to develop an experimental protocol emerged from the difficulty to test several questions in the field (the environement is very variable and the life history of individuals is unkknown) and also by the limitations of the classical protocols ignoring trans-generational effects. In fact, few studies focussed on either the maternal effect on copepods (Ianora et al. 2004) or the trans-generation effect (Lee et al. 2007). On the contrary, the literature in terrestrial ecology provides good examples on the importance of following several generations in the laboratory (Beldade et al., 2002; Klok et al. 2009). However, in all studies, whatever in tererstrial or aquatic ecosystems, building such heavy multigenerational protocols is not always an easy task. For that reason, the first examples using multigenational information was based on the bacterium *Eschirechia coli* (and other similar species) that can develop fast without any heavy logistic support (Bennet et al., 1990). In copepods, maintaining several generations in stable conditions is a real challenge that requires a good understanding of the life cycle of the species and also good knowledge on algal cultures used for feeding. Moreover, observing all individuals for counting and also for examining their morphology required a heavy investment. For example, we showed that the accurate experimental protocol developed here allowed us to identify with precision any unexpected incident like the low food quality event occurred during few days (Souissi et al. 2010). But how many accidents occurred in the previous experiments dealing with copepods leading to discard the results or to publish them ignoring the possible biases? The multigenerational protocol proposed here was a good compromise between the heavy individual observations (Ban 1994; Devreker et al., 2007; 2009) and batch approaches based on the use of individuals from stock culture of copepods. Recently, Brown and Shine (2009) demonstrated that the number of individuals used to initiate a cohort may have an importance on the results obtained. Copepods are adapated to live in groups reaching high numbers, particularly for *E. affinis*, so the effect of density should be considered. We showed, in the first chapter, that the protocol proposed can generate density-dependent mechanisms and their consequences on the growth and fecundity of adult females.

The goal of this protocol was to well acclimat copepods, during several generations, to both cold and high temperatures and we found that *Eurytemora affinis* followed the general temperature size rule (Atkinson 1994). In addition to the mechanisms leading to explain the high performance of *E. affinis* from the Seine estuary at high temperature, we surprisingly demonstrated that, even after long acclimation to a low temperature, a severe heat shock (transfer from 7°C directly to 24°C) was not lethal for this population. We suppose that the low temperature strategy giving large females producing large clutches contributed to this resistance. Moreover, it seemed that the size of females was heritable which is not trivial in copepods.

The standardized protocol applied in a common garden experiment to three different populations of *E. affinis* led to new results. For example, the low fecundities recorded in Gironde estuary (David et al., 2007) were overcome in the laboratory, when the food was available. This means that *E. affinis*, in Gironde, is limited by local unfavorable conditions. Consequently, using only field data compiled from different studies will not be adequate to compare different populations of *E. affinis*. In spite of this standardization, some differences between populations were identified. The most significant one corresponded to the sex-ratio. The Seine population showed a male biased sex-ratio that persisted in both field and laboratory conditions. We can, thus, hypothesize that the sex-ratio has a genetic origin, at least in the Seine population. Conserning the other life cycle traits, we showed that the population from the St Lawrence salt marsh approached two times the extinction. This means that this population maintained for several years in the laboratory became sensitive and probably some selection occurred. We could not identify the direction of this selection because it occurred in a mass culture, but we can suggest that the population maintained at relatively stable temperature (10-15°C) suffered more from the temperature increase. We can show that the response of the Seine population to low food quality episode was different and opposite to that of other populations (mother and grandmother effect responses). Moreover, the intersexual individuals provoked by this event were only observed on the Seine population and at the low temperature (Souissi et al., 2010). This suggests that the determinism of sexes in the Seine population could be different from the other tested populations.

In this study, we also proved that *E. affinis* ovigerous females were capable to adjust their egg size in addition to their clutch size. This variability of these two reproductive traits was associated to female body size. But, when the maternal effect was removed, (i.e. the effect of body size) we found that females were capable to make a trade-off between size and number of offspring depending on the environmental conditions. This study is the first experimental test on the application of Smith and Fretwell model to an egg-bearing copepod and showed its validity for *E. affinis*. The extrapolation of our results, in an ecological context, fitted well to the seasonality of *E. affinis* from the Seine estuary. Then, we generalized our conclusions by applying the same approach to 10 populations from Europe and North America. In addition to the existing genetic separation between the transatlantic populations of *E. affinis*, the clear differences in the general morphological traits and also in the reproductive tactics questioned about the status of these populations among a species complex. We are probably at the boarder (or close to it) of the speciation process. More detailed morphological systematic observations of the transatlantic populations of *E. affinis* confirmed the morphological differences and proposed a clear speciation within this cryptic species (see Alekseev and Souissi, 2011, in appendix).

Moreover, a new consideration of the egg packing constraint allowed us to suggest a new index based on the environemental conditions encountered by *E. affinis* populations whatever their geographical location. Our results suggest that the packing constraints analysis could be used as an indicator of the habitat quality of *E. affinis* populations within and between different estuaries.

This protocol allowed us to explore another angle of the behavioural reproduction in *E. affinis* by elucidating the role of the male and the implication of the female on the mating choice and the sexual selection on the reproductive strategy. We showed here, for the first time, a positive relationship between male and female body sizes and their respective investment in the reproduction. We also made the first evidence of possible advantage of polyandry in the copepod *E. affinis* because females were able to benefit from their mate choices (with a single or multiple partners).

In the last chapter of this thesis, we demonstrated that the different approaches adopted allowed to detect uncommon events that most often are unrecorded. Moreover, in order to study the effects of some punctual events such as the epibiosis relationship, another strategy was applied and permetted to test the effect of the presence of epibiont on the mating behaviour of *E. affinis*.

This thesis answered several questions and provided novel results, not only in terms of copepod ecology but also in terms of general ecology and evolutionary studies. Furthermore, several new scientific questions emerged from this thesis and will lead to the following key perspectives.

Primary, we confirmed in this thesis, for the first time, that the contribution of *E. affinis* male in the reproductive success of *E. affinis* from the Seine estuary is very important. We linked between individual behavioral observations and their consequences at the population level. In fact, males could assess the reproductive status of a female and enhance their mating success. Consequently, the mating in *E. affinis* (and in copepods in general) seemed to be more complex than the simple encounter processes supposed to be modulated by the physical properties of the aquatic environments and the swimming speeds of both sexes. Because the female seemed to get benefit from multiple mating, which was supposed to be waste of energy in most earlier studies, future examinations of these processes is needed to elucidate the exact mechanisms involved in *E. affinis* mating and reproductive strategies.

In general, the multi-generational experimental protocol used in this thesis was appropriate to test several ecological questions. The protocol can be thus generalized to study the effects of any other external factor on the plasticity of *E. affinis*. Because this population lives in highly polluted estuaries (i.e. in the Seine estuary) the priority should be given to test the effect of different classes of pollutants on the life cycle traits of *E. affinis*. Recent papers used more than one generation of copepods in ecotoxicological studies but the protocols applied are based on individual observations and varied from one study to another. The use of a standardized protocol, as developed in this thesis, will offer a common framework to compare all results obtained in different experimental conditions. Moreover, as the mating behavior has been identified as a critical mechanism in the reproduction, any perturbation of the swimming/mating behavior of *E. affinis* (as shown when epibionts were present) will hamper the reproduction and consequently the fitness of the population. Because the injection of a pollutant (alkylphenol) disrupted the swimming behavior of *E. affinis* males and females (Cailleaud et al., 2011) in a similar way to what we observed when epibiont are present, we suggest that future ecotoxicological studies should also focus on the mating success.

Our results showed clear differences in the reproductive strategies of *E. affinis* in Europe and North America. We suggested that the shift in the reproductive strategy between transatlantic populations could be due to evolutionary processes. We also suggested that the packing constraints (and also other indices) could be used to track the suitability of *E. affinis* habitats (and their qualities). All recent observations confirmed that *E. affinis* in the Scheldt estuary responded positively to the improvement of water quality in the upper (freshwater) part of this estuary (Mialet et al. 2010). Our results confirmed that the shift of *E. affinis* population in the freshwater zone in the Scheldt estuary was accompanied by an improvement of its reproductive status. In fact, *E. affinis* in the Scheldt, Elbe (where the population can be observed in the very low salinity zone) and also the Chesapeake Bay seemed to develop without heavy environmental constraints. Whereas, in the Gironde estuary, the reproduction of *E. affinis* seemed to be highly limited. In order to separate between genetic and environmental processes, it should be interesting to develop common garden experiments using a population of *E. affinis* isolated from the Scheldt estuary (Freshwater zone). Several hypotheses can be tested and the role of *E. affinis* as bioindicator of water quality (in macrotidal estuaries) could be generalized.

Finally, this thesis was inspired from several works developed in terrestrial ecology and particularly using key biological models. We confirmed that the same ecological/evolutionary framework can be applied to both aquatic and terrestrial ecosystems. For example, the multigenerational experimental approach, common in terrestrial ecology, was successfully adapted in this work to an aquatic organism. This study is pioneer in aquatic ecology and should reinforce the interconnections between both disciplines. Several studies in the theoretical framework (i.e., temperature size rule, optimal reproduction theory, significance of trade-off in ecology, acclimation, selection, etc.) could be applied to *E. affinis* and copepods in general. We should reinforce the promotion of this species complex as a biological model for multidisciplinary studies. We should use more genetic tools to track the multiple paternity suspected here and test its consequences on both life cycle strategies and evolution of the species. At large scale, it is important to confirm in the future the process of speciation in this biological group and enlarge the studies to other congeneric groups of *E. affinis* characterized by different evolutionary processes. All these studies should include detailed examination of the reproductive strategy that is a key mechanism in the ecology and evolution of all organisms.

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Appendix

A new species within the Eurytemora affinis complex (Copepoda: Calanoida) from the Atlantic Coast of USA with observations on eight morphologically different European populations

VICTOR R. ALEKSEEV[1] AND ANISSA SOUISSI[2,3,4]

[1]*Zoological Institute, Russian Academy of Sciences, Universitetskaya Emb. 1, 199034 St. Petersburg, Russia. Email: alekseev@zin.ru*

[2] *Université Lille Nord de France, F-59000 Lille, France.*

[3] *USTL, LOG, Station Marine de Wimereux, 28 av. Foch, F-62930 Wimereux, France.*

[4] *CNRS-UMR 8187, LOG, F-62930 Wimereux, France.*

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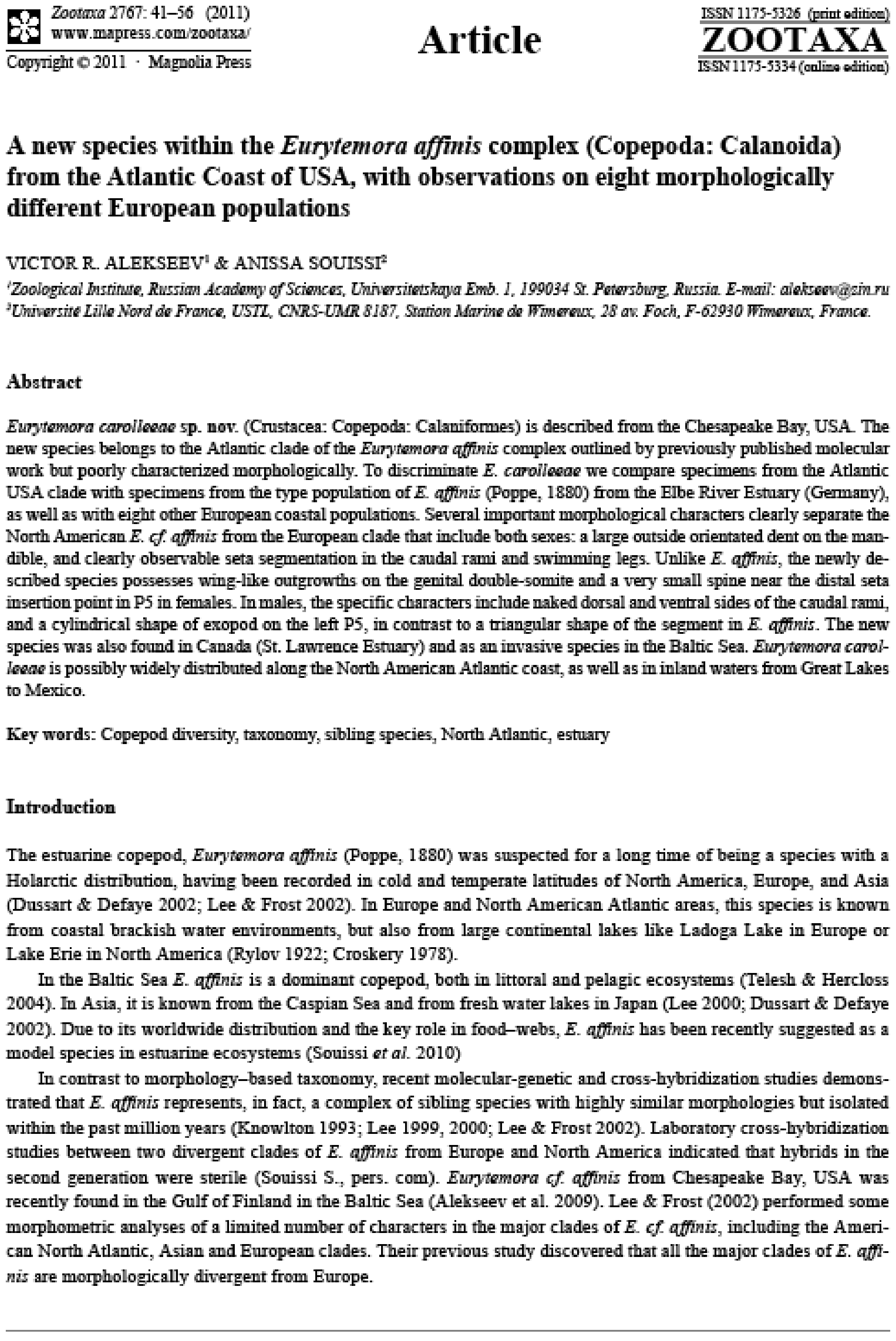

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A new species within the *Eurytemora affinis* complex (Copepoda: Calanoida) from the Atlantic Coast of USA, with observations on eight morphologically different European populations

VICTOR R. ALEKSEEV[1] & ANISSA SOUISSI[2]
[1]*Zoological Institute, Russian Academy of Sciences, Universitetskaya Emb. 1, 199034 St. Petersburg, Russia. E-mail: alekseev@zin.ru*
[2]*Université Lille Nord de France, USTL, CNRS-UMR 8187, Station Marine de Wimereux, 28 av. Foch, F-62930 Wimereux, France.*

Abstract

Eurytemora carolleeae **sp. nov.** (Crustacea: Copepoda: Calaniformes) is described from the Chesapeake Bay, USA. The new species belongs to the Atlantic clade of the *Eurytemora affinis* complex outlined by previously published molecular work but poorly characterized morphologically. To discriminate *E. carolleeae* we compare specimens from the Atlantic USA clade with specimens from the type population of *E. affinis* (Poppe, 1880) from the Elbe River Estuary (Germany), as well as with eight other European coastal populations. Several important morphological characters clearly separate the North American *E. cf. affinis* from the European clade that include both sexes: a large outside orientated dent on the mandible, and clearly observable seta segmentation in the caudal rami and swimming legs. Unlike *E. affinis*, the newly described species possesses wing-like outgrowths on the genital double-somite and a very small spine near the distal seta insertion point in P5 in females. In males, the specific characters include naked dorsal and ventral sides of the caudal rami, and a cylindrical shape of exopod on the left P5, in contrast to a triangular shape of the segment in *E. affinis*. The new species was also found in Canada (St. Lawrence Estuary) and as an invasive species in the Baltic Sea. *Eurytemora carolleeae* is possibly widely distributed along the North American Atlantic coast, as well as in inland waters from Great Lakes to Mexico.

Key words: Copepod diversity, taxonomy, sibling species, North Atlantic, estuary

Introduction

The estuarine copepod, *Eurytemora affinis* (Poppe, 1880) was suspected for a long time of being a species with a Holarctic distribution, having been recorded in cold and temperate latitudes of North America, Europe, and Asia (Dussart & Defaye 2002; Lee & Frost 2002). In Europe and North American Atlantic areas, this species is known from coastal brackish water environments, but also from large continental lakes like Ladoga Lake in Europe or Lake Erie in North America (Rylov 1922; Croskery 1978).

In the Baltic Sea *E. affinis* is a dominant copepod, both in littoral and pelagic ecosystems (Telesh & Hercloss 2004). In Asia, it is known from the Caspian Sea and from fresh water lakes in Japan (Lee 2000; Dussart & Defaye 2002). Due to its worldwide distribution and the key role in food–webs, *E. affinis* has been recently suggested as a model species in estuarine ecosystems (Souissi *et al.* 2010)

In contrast to morphology–based taxonomy, recent molecular-genetic and cross-hybridization studies demonstrated that *E. affinis* represents, in fact, a complex of sibling species with highly similar morphologies but isolated within the past million years (Knowlton 1993; Lee 1999, 2000; Lee & Frost 2002). Laboratory cross-hybridization studies between two divergent clades of *E. affinis* from Europe and North America indicated that hybrids in the second generation were sterile (Souissi S., pers. com). *Eurytemora cf. affinis* from Chesapeake Bay, USA was recently found in the Gulf of Finland in the Baltic Sea (Alekseev et al. 2009). Lee & Frost (2002) performed some morphometric analyses of a limited number of characters in the major clades of *E. cf. affinis*, including the American North Atlantic, Asian and European clades. Their previous study discovered that all the major clades of *E. affinis* are morphologically divergent from Europe.

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The results of our analyses allow us to support the opinion of Lee (2000) on the possible validity of the Atlantic clade of *E. cf. affinis* from the USA as a new species. The present study is the first description of a cryptic species within this *E. affinis* complex.

Material and methods

The type material for this new species was selected from a sample collected on 16 April 2008 by Dr. D. Kimmel (East Carolina University, USA) in the Chesapeake Bay, Atlantic coastline of USA. We also used material from the St. Lawrence Estuary, Canada donated by Dr. G. Winkler (ISMER, University of Québec at Rimouski, Canada). For comparison we used specimens from The Elbe, Germany (*terra typica* for *Eurytemora affinis* (Poppe, 1880) collected in 2006 twice per season by Dr. Winkler and Dr. Boillens and preserved in the Marine Station of Wimereux, University of Lille 1, France (Prof. S. Souissi collection). Individuals from eight other European populations of *E. affinis* were also collected by the authors in 2008 and 2009 (Table 1 and Fig. 1).

TABLE 1. *Eurytemora* sampling sites in Europe and North America.

Country, area	Locality/№ on the map	Date
USA, Atlantic coast	Chesapeake Bay/1	April 2008
Canada, Quebec	R. St. Lawrence estuary/2	July 2008
Germany, Hamburg	R. Elbe estuary/3	March 2006
Spain, Seville City	R. Guadalquivir/11	January 2009
France, Atlantic coast	R. Gironde estuary/6	April 2006
France, Atlantic coast	R. Loire estuary/5	June 2009
France, Atlantic coast	R. Seine estuary/4	July 2009
Belgium, North Sea coast	R. Schelde estuary/7	April 2006
Latvia Baltic Sea coast	Riga Bay/8	June 2008
Finland Baltic Sea, Helsinki	City Fish Port /9	August 2009
Russia, Baltic Sea, Gulf of Finland	R. Luga estuary/10	August 2008

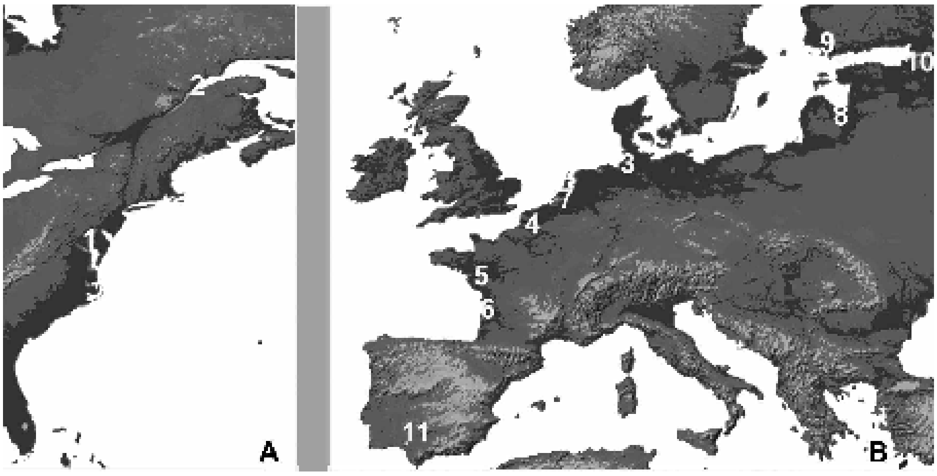

FIGURE 1. Sampling map of *Eurytemora carolleeae* **sp. nov.** in North America (A) and *Eurytemora affinis* (Poppe, 1880) in Europe (B).

The samples were sorted in the laboratory under a dissection microscope (Olympus, SZX2), and about 60 adults from each population were selected for analyzes. Before dissection, copepod adults were photographed with a digital camera and measured with an ocular micrometer (5 µm resolution). After dissection, the individuals were placed on slides in pure glycerol, covered with a cover slip and ringed with Canadian balsam. The slides were then observed at maximum resolution up to 1000 x (Plan objective 100 x, oil immersion) under a compound microscope (Zeiss IMAGER) equipped with Nomarski system for differential interference contrast microscopy and a drawing tube. Initial pencil drawings were converted to India ink and placed on A4 size tracing paper. The tables were scanned at a resolution of 600 dpi, numbered and reorganized with a computerized graphics program (Adobe Photoshop 7).

To delineate the species, all morphological characters were checked in both sexes, including not only secondary sexual dimorphic characters typically used in copepod taxonomy, but also mouth appendages, micro characters of the fifth legs, seta structure and body shape.

To observe variation between *E. carolleeae* **sp. nov.** and *E. affinis* (Poppe, 1880), as well as among 8 local populations of *E. affinis* in Europe, we measured for both sexes caudal rami length and width; in the swimming legs 1 and 4 the distal exopod segment and distal spine lengths were also measured.

In females, we measured additionally leg 5 exopod spine lengths, and for the genital double-somite we determined the length and width in the anterior (W1) and posterior (W2) sides of the somite (Fig 2). For males, length and maximal width of the first segment of leg 5 exopod were measured. Statistical analyses were performed in Statistica-7 program.

The type slides for *E. carolleeae* were placed in the type collection of the Zoological Institute of the Russian Academy of Sciences, St. Petersburg, Russia (ZIN RAN) under reference numbers 55052–55054. Slides with dissected individuals from 8 European populations of *E. affinis,* including the type population from the Elbe River, are stored in the Copepod division of the Russian Federal Collection N 96–03–16.

For terminology we mainly followed Huys & Boxshall (1991). Abbreviations used are: END, endopod; EXP, exopod; BAS, basipod; P1–P4, swimming legs 1–4; P5, leg 5.

Systematics

Class Copepoda H. Milne Edwards, 1840

Order Calanoida Sars, 1903

Superfamily Diaptomoidea Baird, 1850

Family Temoridae Giesbrecht, 1893

Genus *Eurytemora* Giesbrecht, 1881

Eurytemora carolleeae sp. nov.
(Figs 2–7, 8A, 9A–D)

Type material. Holotype, ZIN RN 55052, a female dissected on 1 slide; from the Chesapeake Bay, USA (39°23.81'N, 76°03.32'W), collected by Dr Kimmel, 16 April 2008.

Allotype, ZIN RN 55053, a male dissected on 1 slide; from the Chesapeake Bay, USA (39°23.81'N, 76°03.32'W).

Other paratypes, ZIN RN 55054/1–10, 15 females and 15 males from the Chesapeake Bay, USA (39°23.81'N, 76°03.32'W), dissected on 10 slides (3 individuals per 1 slide)

Description. Female (Figs. 2–5). Body transparent, genital double-somite yellowish brown. Length measurements: Full body length without caudal setae 1160 µm, with caudal setae 1655 µm.; cephalosome 418 µm and 4 free thoracic segments 1/2/3/4 = 90/70/70/40 µm. Urosome 460 µm, genital double-somite 130 µm, 3 following somites 1/2/3 = 80/60/60 µm and caudal rami 130 µm.

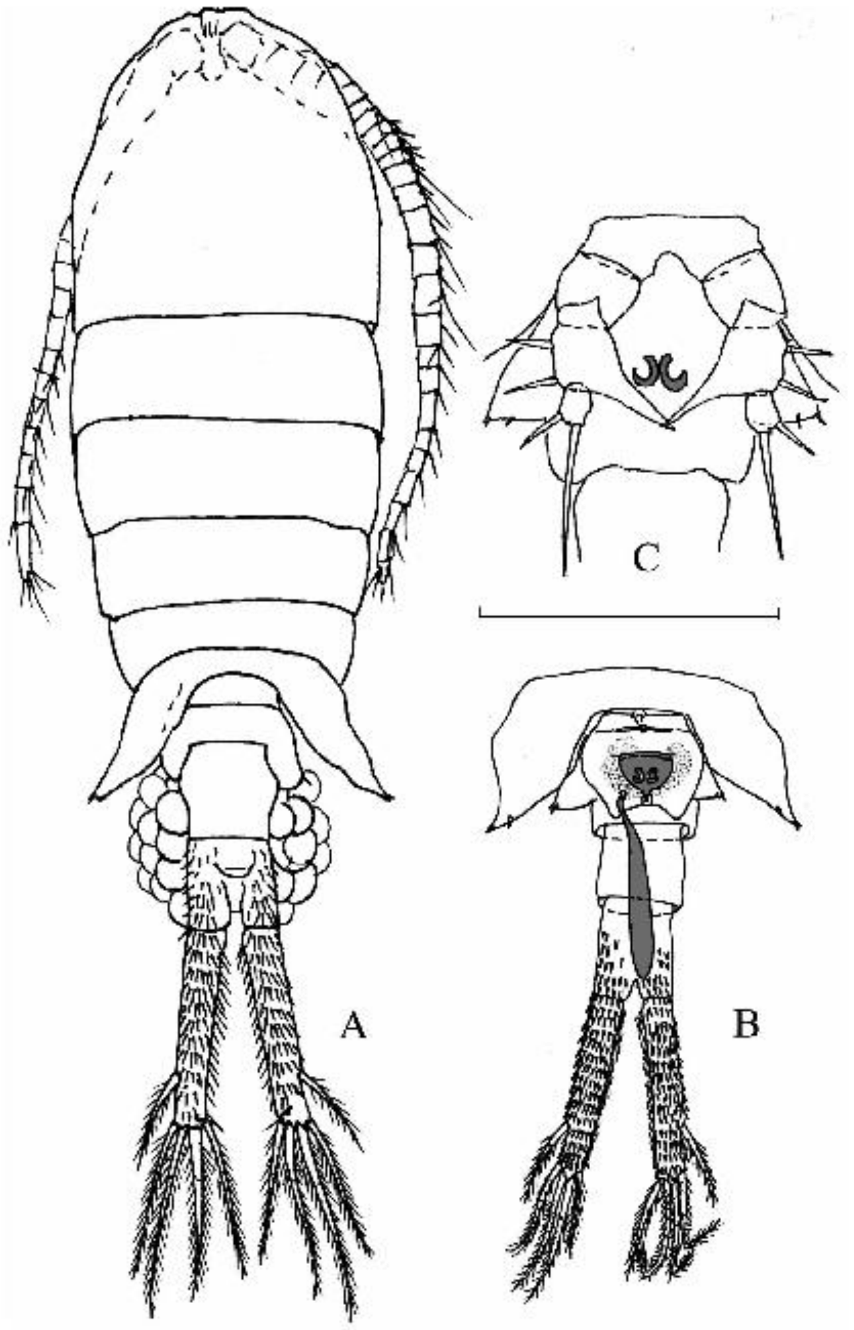

FIGURE 2. *Eurytemora carolleeae* **sp. nov.**, female, ZIN 55050 (holotype): A, habitus, dorsal view; B, urosome, ventral view; C, genital double-somite with P5, ventral view. Scale bar: A, B, 300 µm; C, 150 µm.

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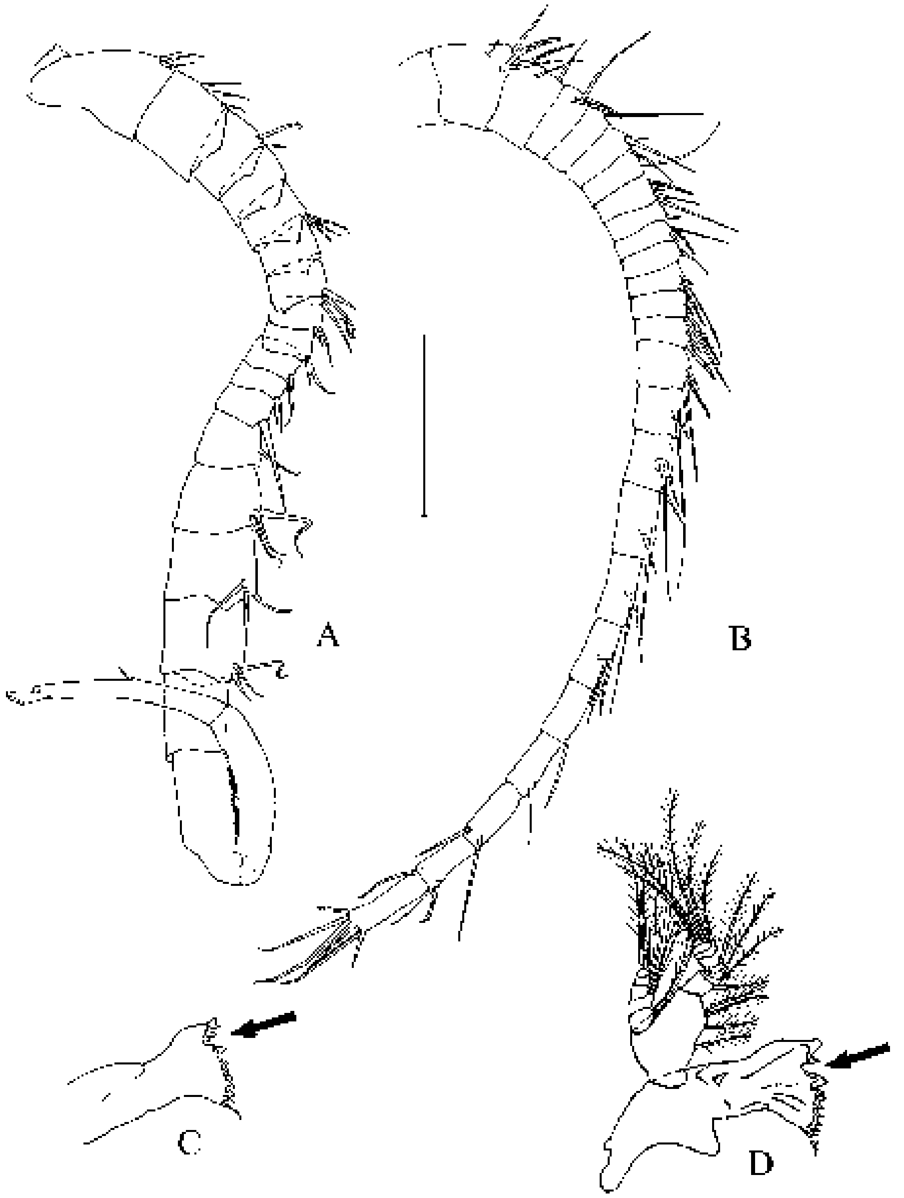


FIGURE 3. *Eurytemora carolleeae* sp. nov. A, male antennula; B, female left antennula; C, male gnathobasis of mandible; D, female mandible with palp. Arrows indicate separate processor on gnathobasis. Scale bar: A, B, 125μm; C, D, 62.5 μm.

Cephalothorax (Fig. 2A) as long as wide, with maximum width close to middle, frontal part of cephalothorax oval.

Last thoracic somite with 1–2 small spines on wing-like outgrowths of lateral margin.

Genital double-somite 1.5 times as wide as long, due to wing-like outgrowths in anterior part of the somite, with two relatively long spines on both sides, with seminal receptacle as shown in Figs. 2B–C.

Caudal rami (Fig. 2B) divergent, 6.2 times as long as wide, with long and strong hair-setae on both sides, as well as on last abdominal somite.

Length proportions of terminal setae, beginning from outermost caudal seta:1/1.3/1.15/1. Length proportions of dorsal and lateral setae to outermost seta 0.25 and 0.6 times respectively. All caudal setae with clearly observable segment-like divisions.

Antennules (Fig. 3A) of 25 segments, reaching end of third free thoracic somite, setation of segments beginning from first segment: 0/4/4/3/1/3/1/4/1/3/1/3/3/2/3/2/3/2/2/3/1/1/2/3/7. Most segments with 2 and more setae provided with 1 aesthetasc, last (distal) segment with 6 setae and 1 aesthetasc.

Antenna (Fig. 4A) biramous, composed of 2-segmented protopod, 2-segmented endopod and 6-segmented exopod. First exopod segment with 3 setae, second segment with 2 setae, 3–5 segments with 1 seta each, 6 (distal) segment with 4 setae. First endopodal segment with 2 setae, second with 9 setae laterally and 7 setae at distal end.

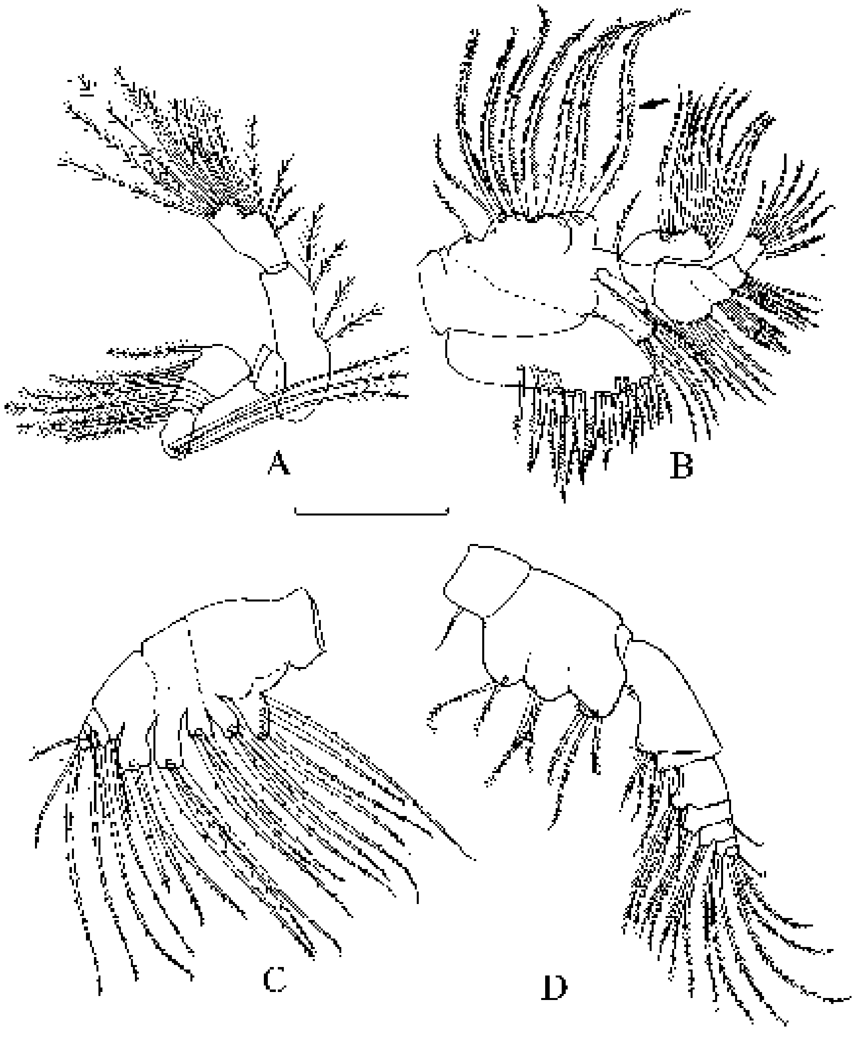


FIGURE 4. *Eurytemora carolleeae* **sp. nov.**, female, ZIN 55050 (holotype): A, antenna; B, maxillula, ventral view; C, maxilla; D, maxilliped. Scale bar: 50 µm. Arrow indicating seta segmentation.

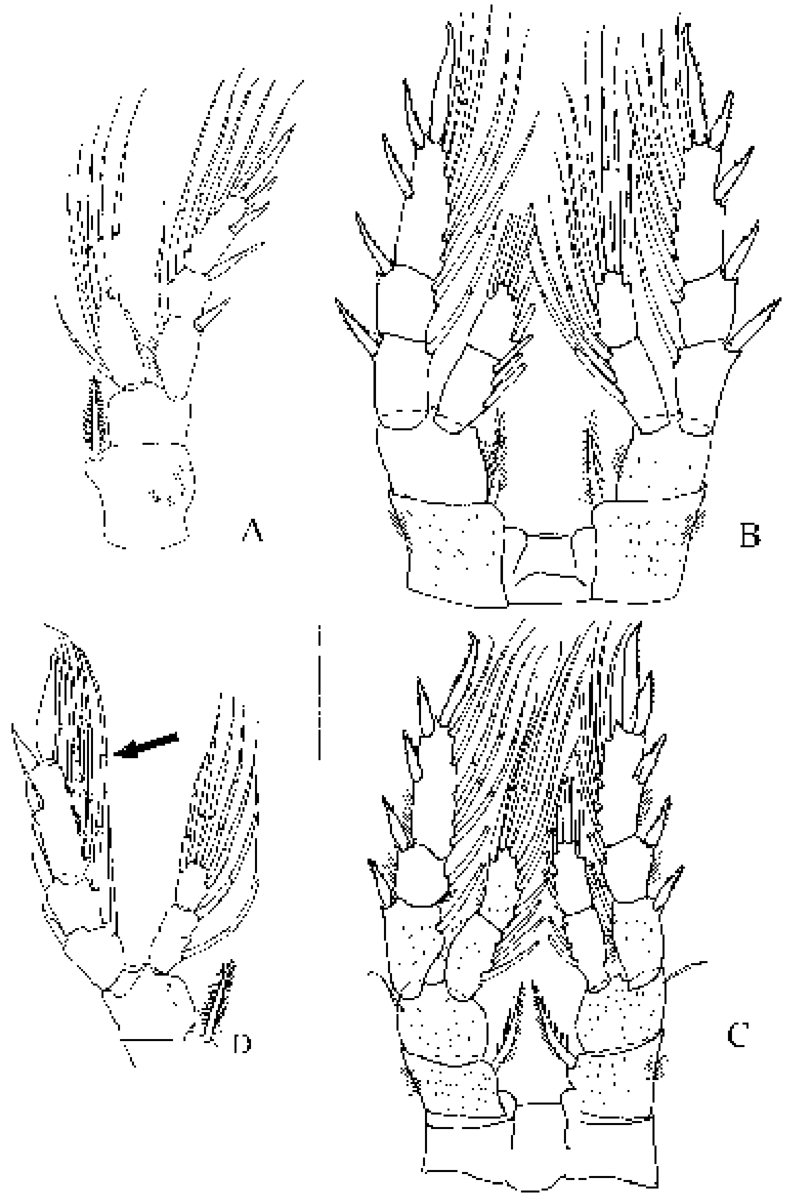
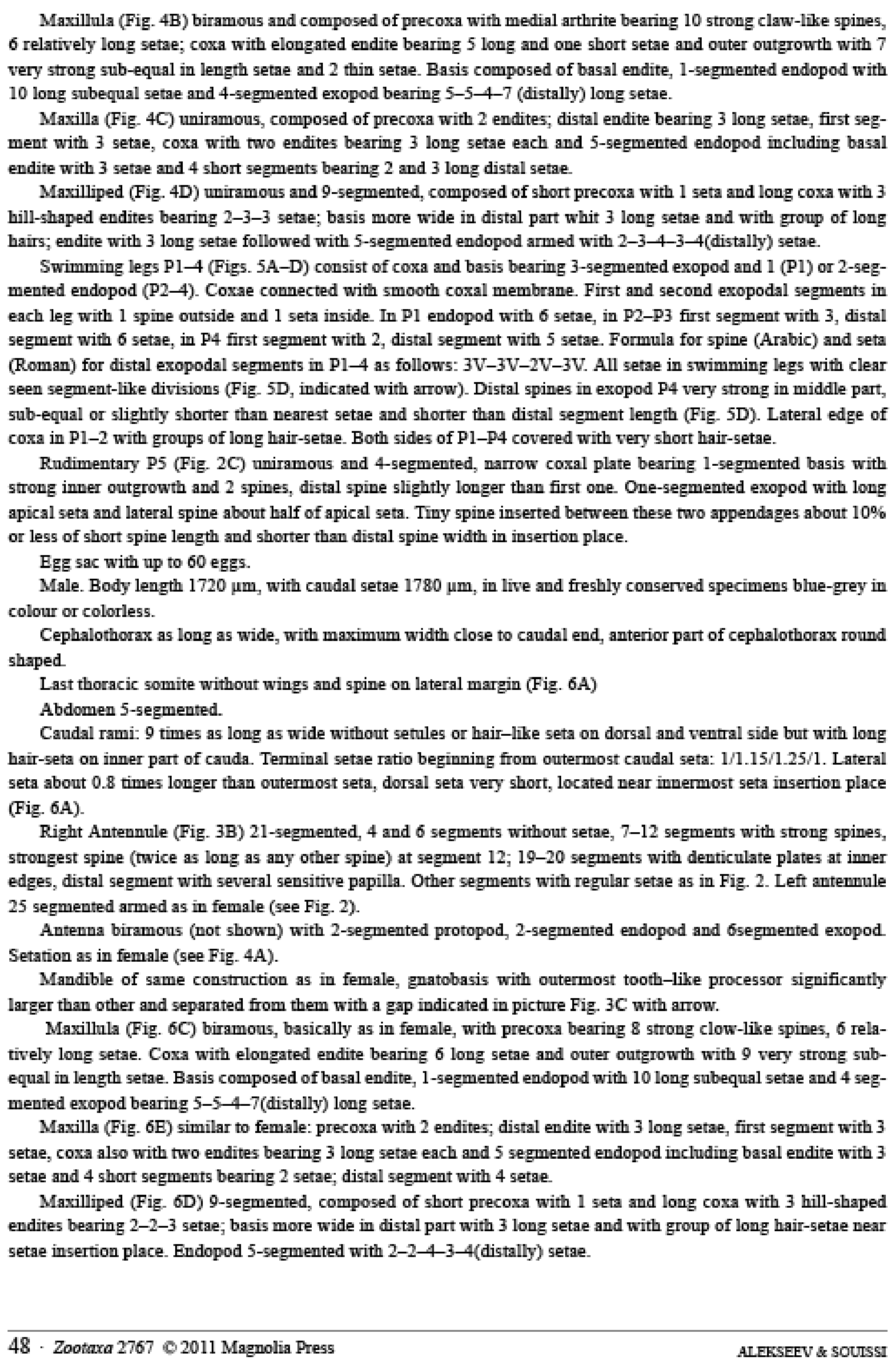


FIGURE 5. *Eurytemora carolleeae* **sp. nov.**, female, ZIN 55050 (holotype): A, left swimming leg 1, anterior view; B, swimming legs 2, anterior view; C, swimming legs 3, anterior view; D, swimming leg 4, anterior view. Scale bar: 100 µm. Arrow indicating seta segmentation.

Mandible (Fig. 3D) composed of coxa with gnatobasis, one of its tooth (outermost) significantly larger than other and separated from them. Coxa in middle with biramous mandibular palp, basis with 4 setae, 8-segmented exopod and 5-segmented endopod. Distal segment of exopod with two sub-equal setae, other segments bearing single long seta each. Distal segment of endopod with 2 long setae and 1 short seta, other segment with 2 long setae each.



Maxillula (Fig. 4B) biramous and composed of precoxa with medial arthrite bearing 10 strong claw-like spines, 6 relatively long setae; coxa with elongated endite bearing 5 long and one short setae and outer outgrowth with 7 very strong sub-equal in length setae and 2 thin setae. Basis composed of basal endite, 1-segmented endopod with 10 long subequal setae and 4-segmented exopod bearing 5–5–4–7 (distally) long setae.

Maxilla (Fig. 4C) uniramous, composed of precoxa with 2 endites; distal endite bearing 3 long setae, first segment with 3 setae, coxa with two endites bearing 3 long setae each and 5-segmented endopod including basal endite with 3 setae and 4 short segments bearing 2 and 3 long distal setae.

Maxilliped (Fig. 4D) uniramous and 9-segmented, composed of short precoxa with 1 seta and long coxa with 3 hill-shaped endites bearing 2–3–3 setae; basis more wide in distal part whit 3 long setae and with group of long hairs; endite with 3 long setae followed with 5-segmented endopod armed with 2–3–4–3–4(distally) setae.

Swimming legs P1–4 (Figs. 5A–D) consist of coxa and basis bearing 3-segmented exopod and 1 (P1) or 2-segmented endopod (P2–4). Coxae connected with smooth coxal membrane. First and second exopodal segments in each leg with 1 spine outside and 1 seta inside. In P1 endopod with 6 setae, in P2–P3 first segment with 3, distal segment with 6 setae, in P4 first segment with 2, distal segment with 5 setae. Formula for spine (Arabic) and seta (Roman) for distal exopodal segments in P1–4 as follows: 3V–3V–2V–3V. All setae in swimming legs with clear seen segment-like divisions (Fig. 5D, indicated with arrow). Distal spines in exopod P4 very strong in middle part, sub-equal or slightly shorter than nearest setae and shorter than distal segment length (Fig. 5D). Lateral edge of coxa in P1–2 with groups of long hair-setae. Both sides of P1–P4 covered with very short hair-setae.

Rudimentary P5 (Fig. 2C) uniramous and 4-segmented, narrow coxal plate bearing 1-segmented basis with strong inner outgrowth and 2 spines, distal spine slightly longer than first one. One-segmented exopod with long apical seta and lateral spine about half of apical seta. Tiny spine inserted between these two appendages about 10% or less of short spine length and shorter than distal spine width in insertion place.

Egg sac with up to 60 eggs.

Male. Body length 1720 µm, with caudal setae 1780 µm, in live and freshly conserved specimens blue-grey in colour or colorless.

Cephalothorax as long as wide, with maximum width close to caudal end, anterior part of cephalothorax round shaped.

Last thoracic somite without wings and spine on lateral margin (Fig. 6A)

Abdomen 5-segmented.

Caudal rami: 9 times as long as wide without setules or hair–like seta on dorsal and ventral side but with long hair-seta on inner part of cauda. Terminal setae ratio beginning from outermost caudal seta: 1/1.15/1.25/1. Lateral seta about 0.8 times longer than outermost seta, dorsal seta very short, located near innermost seta insertion place (Fig. 6A).

Right Antennule (Fig. 3B) 21-segmented, 4 and 6 segments without setae, 7–12 segments with strong spines, strongest spine (twice as long as any other spine) at segment 12; 19–20 segments with denticulate plates at inner edges, distal segment with several sensitive papilla. Other segments with regular setae as in Fig. 2. Left antennule 25 segmented armed as in female (see Fig. 2).

Antenna biramous (not shown) with 2-segmented protopod, 2-segmented endopod and 6segmented exopod. Setation as in female (see Fig. 4A).

Mandible of same construction as in female, gnatobasis with outermost tooth–like processor significantly larger than other and separated from them with a gap indicated in picture Fig. 3C with arrow.

Maxillula (Fig. 6C) biramous, basically as in female, with precoxa bearing 8 strong clow-like spines, 6 relatively long setae. Coxa with elongated endite bearing 6 long setae and outer outgrowth with 9 very strong subequal in length setae. Basis composed of basal endite, 1-segmented endopod with 10 long subequal setae and 4 segmented exopod bearing 5–5–4–7(distally) long setae.

Maxilla (Fig. 6E) similar to female: precoxa with 2 endites; distal endite with 3 long setae, first segment with 3 setae, coxa also with two endites bearing 3 long setae each and 5 segmented endopod including basal endite with 3 setae and 4 short segments bearing 2 setae; distal segment with 4 setae.

Maxilliped (Fig. 6D) 9-segmented, composed of short precoxa with 1 seta and long coxa with 3 hill-shaped endites bearing 2–2–3 setae; basis more wide in distal part with 3 long setae and with group of long hair-setae near setae insertion place. Endopod 5-segmented with 2–2–4–3–4(distally) setae.

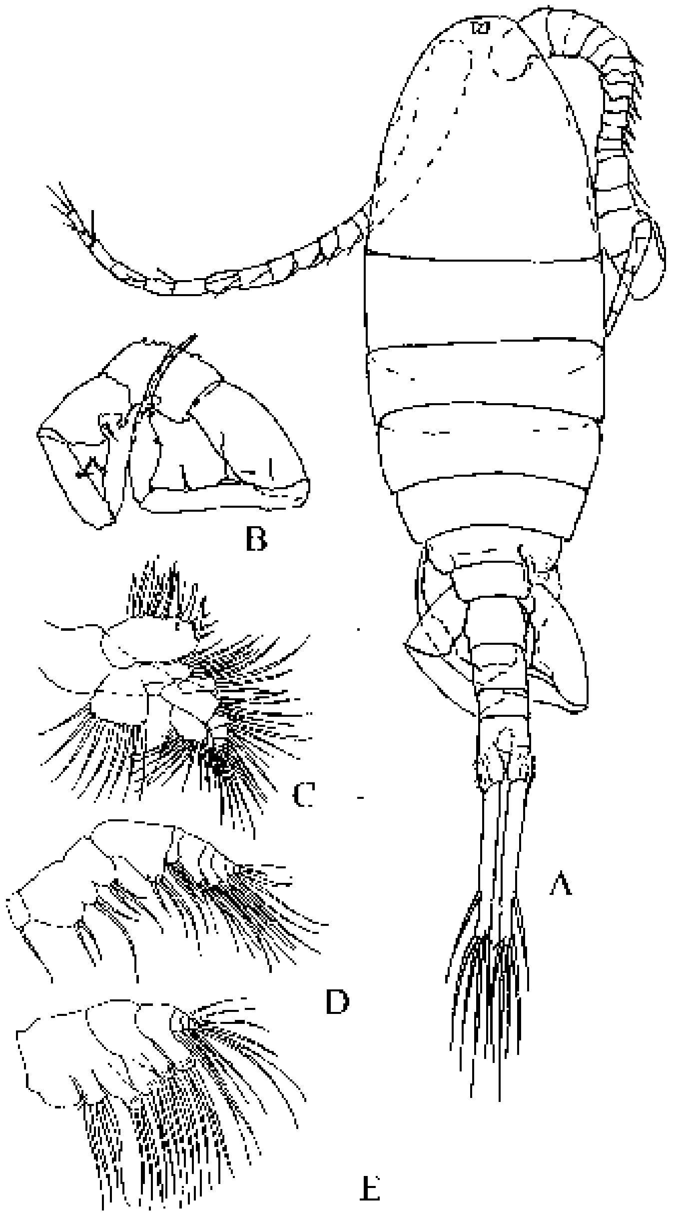


FIGURE 6. *Eurytemora carolleeae* **sp. nov.**, male, ZIN 55051 (paratype): A, habitus, dorsal view; B, leg 5, anterior view; C, maxillula, anterior view; D, maxilliped, anterior view; E, maxilla, anterior view. Scale bar: A, 325 µm; B, 250 µm; C– E, 125 µm.

Swimming legs P1–4 (Figs. 7A–D) constructed basically like in female. Formula for spine (Arabic) and seta (Roman) for distal exopod segments in P1–4 as follows: 3V–3V–2V–3V. All setae in swimming legs with clear seen segment-like divisions. Distal spines in exopod P1–4 not so strong in middle part as in female, slightly shorter than nearest setae and longer than distal segment length (Fig. 7D). Lateral edge of coxa in P1–4 with groups of long hair-setae. Both sides of P1–P4 covered with very short hair-setae.

Rudimentary legs P5 (Fig. 6B): right leg with basipodal segment cylindrical in shape and with small hill on inner side pointed with long spine, distal bent segment with several sensitive pores and two short spines in middle. Left leg basipod also cylindrical about 1.45 times as long as wide, next exopodal segment with two long spine in middle part, distal segment with strong long spine in middle and hook in end similar in construction to E. affinis.

Etymology. The new species is named in honor of Dr. Carol Lee, Wisconsin University, Madison, USA, in recognition of her pioneering work on cryptic species within the *E. affinis* complex.

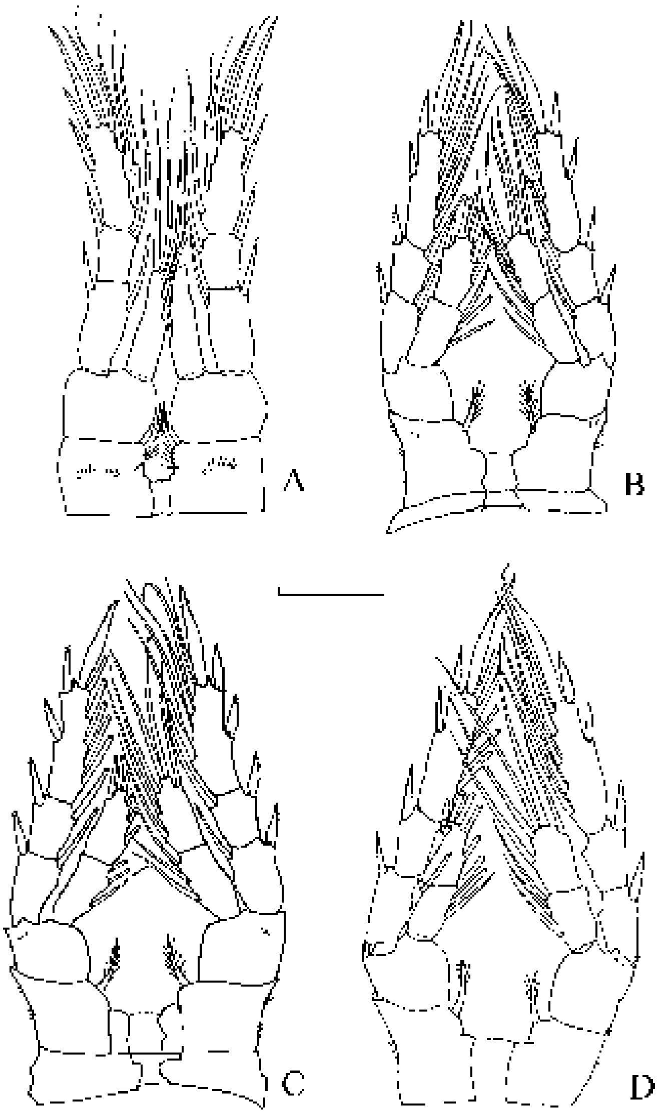


FIGURE 7. *Eurytemora carolleeae* **sp. nov.**, male, ZIN 55051 (paratype): A , swimming leg 1, anterior view; B, swimming leg 2, anterior view; C, swimming leg 3, anterior view; D, swimming leg 4, anterior view. Scale bar: 100 µm.

Remarks. The new species was discriminated from *E. affinis* (Poppe, 1880) that inhabits European coastal brackish and fresh water habitats. ***Eurytemora carolleeae* sp. nov.** differs from *E. affinis* by a combination of characters that includes in both sexes: clearly seen segment-like divisions in setae on P1–P4 and caudal rami; mandible with large outside orientated tooth separated with a gap from other teeth (Figs. 3A, 9).

Females ***E. carolleeae*** are equiped with wing-like outgrowths of the genital double-somite that significantly increases the proportion between anterior and posterior parts of the somite (Fig. 9C; Table 2). This species has P5 with a very small (tiny) spine in the second exopodal segment placed between two distal spines. The length of this tiny spine is less than the width of the nearest spines, or about 10 % of the short distal spine length (Table 2).

TABLE 2. Selected morphometric indexes in females of ***E. carolleeae*** **sp. nov.** and *E. affinis* (Poppe, 1880) from their type localities. Mean + standard deviation (Min–Max). In bold – significant difference with $p<0.05$. *Only in one female among 56 examined, possibly an aberrant specimen.

Species/Indexes	Caudal rami, L/W	Genital somite, W2/W1	Leg 5, tiny spine/spine1	Leg 4, Long spine/Distal Segment
E. carolleeae	6,06+0,531 (5–7,4)	1,54+0,103 (1,35–1,67)	0,11+0,029 (0,06– 0,2*)	0,85+0,068 (0,70– 0,99)
E. affinis	6,08+0,638 (5,2–7,8)	1,24+0,075 (1–1,39*)	0,25+0,053 (0,18– 0,36)	0,94+0,066 (0,8 –1,07)

TABLE 3. Selected morphometric indexes for ***E. carolleeae*** **sp. nov.** and *E. affinis* (Poppe, 1880) in males from the type localities. Mean + standard deviation (Min–Max).

Species/Indexes	Caudal rami, L/W	Leg 5 Basipod left, L/W	Leg 4, Long spine/Distal Segment	Leg 1, Long spine/Distal Segment
E. carolleeae	9,56+0,936 (8,06–11,07)	1,43+0,13 (1,27–1,6)	1,06+0,060 (0,95–1,14)	1,1+0,117(1–1,39)
E. affinis	7,77+1,037 (6–10,45)	0,96+0,049 (0,91–1,08)	1,14+0,052 (1,03–1,25)	1,21+0,081 (1,06–1,38)

TABLE 4. Selected morphometric indexes in female and male ***E. carolleeae*** **sp. nov.** (USA, Chesapeake Bay) and *E. affinis* (Poppe, 1880) (from several European localities). Populations from the type localities are indicated in bold.

Species	AREA	Female		Male	
		Caudal rami, L/W	Leg 5, tiny spine/spine1	Caudal rami, L/W	P5 basipod left, L/W
E. carolleeae	CHESAPEAKE BAY	6,06	0.095	9.56	1.43
E. affinis	RIGA BAY	7.59	0.292	10.86	0.98
E. affinis	SCHELDE	7.03	0.31	8.01	0.92
E. affinis	SEINE	7.61	0.241	8.77	0.94
E. affinis	LOIRE	6.26	0.196	7.93	1.1
E.affinis	ELBE	6,08	0.25	7.77	0.96
E.affinis	GUADALQUIVIR	–	–	12.91	1
E. affinis	GIRONDE	7.87	0.239	7.04	0.98

In males of ***E. carolleeae*** the caudal rami are naked on both dorsal and ventral sides, left rudimentary P5 with exopod of cylindrical shape, and length/width proportion not less than 1.25 times (Table 3, Fig. 9).

In *E. affinis* only a small proportion of adults in some local populations (see below) have setae on swimming legs with unclear segment-like divisions. They have mandible with more or less equal teeth; the outside tooth is not separated from neighboring teeth by a gap.

Female *E. affinis,* in contrast to the newly described species, has a genital somite with only small upper outgrowths (if present) which do not look like wings; the ratio between anterior and posterior parts in the genital somite is significantly less than 1.5 (see Table 2). In P5 the tiny spine is much longer than width of the nearest distal spines and usually is more than 15 % of the short distal spine length (Table 4).

Male *E. affinis* always has caudal rami with at least several spines on the dorsal surface. In P5 the left exopodal segment has a triangular shape outgrowth that decreases length/width proportion in this segment up to 1 or even less (see Table 3).

Variability among European populations of *E. affinis* (Poppe, 1880)

Eurytemora affinis is known for significant morphological variation among individuals from local populations (Dussart 1967). Variability in significant characters in *E. affinis* was checked here and compared among eight distant Atlantic and Baltic populations, inhabiting river estuaries. Hereafter we present brief comments on morphological variations found among eight European populations of *E. affinis*.

Gironde River Estuary, France. About 15% of the population had setae with segment-like divisions in P1–P4. Other signs of *E. affinis* (P5 construction, shape of teeth in mandible) both in male and female were close to the type population from the Elbe River (see Table 2, 4).

Guadalquivir River in Seville, Spain. With exception of the Basque Country (Albaina et al. 2009), this is the only river in Spain where *E. affinis* has been found. In January (date of sampling) we found only adult males and copepodid 5 females; there were no adult females. Males had typical triangular outgrowths on the left basipod of P5, caudal rami were long and narrow (L/W=12,9+1,3), but always with at least a few tiny spines on the dorsal surface of rami.

Helsinki Fishing Port, Finland. In some males and females we found setae with clearly observable segment-like divisions. Caudal rami of males were quite long, about 11 times as long as wide, but always with tiny hairs and dents on the dorsal surface. Other characters were the same as in the type population of *E. affinis* from the Elbe River (see Table 2, 4).

Loire River Estuary, France. More than 50% of the *E. affinis* females examined for swimming legs had setae with segment-like divisions. We also found several females with segment-like division in caudal rami setae similar to *E. carolleeae*. One female had wing-like processes on the genital double-somite similar to American females from the Chesapeake Bay. The tiny spine in P5 in *E. affinis* females in the Loire was less than in the type population (0,196 + 0,035). This index on average was significantly larger than in *E. carolleeae* **sp. nov.** Only in one female from the Loire it was similar to the North-American species. At least some females of *Eurytemora* in The Loire are morphologically close to the North-American species. In several seaports in the Baltic we found an invasion of *E. carolleeae*, and therefore we can suspect a similar penetration of the same species in the Loire estuary also. This assumption should be tested with molecular-genetic tools.

Luga River Estuary, Gulf of Finland, Baltic Sea, Russia (Fig. 8). Here individuals of both taxa were found living together (Alekseev *et al* 2009). *Eurytemora carolleeae* females can be easily separated from *E. affinis* by lower length/width proportions in the genital double-somite and in the caudal rami, and by the frontal part of the cephalothorax being oval in *E. carolleeae* and triangular in *E. affinis* (see Fig. 8). In males, *E. affinis* length/width proportion in the basipod of left P5 was about 1, whereas in *E. carolleeae* it was never less than 1.4.

Riga Bay, Baltic Sea, Latvia. Females of *E. affinis* at this site have the tiny spine on the distal exopodite segment P5 that is about 30% of the nearest small spine 1 length (longest ratio among 8 populations studied). In males, caudal rami have groups of strong denticles on the dorsal surface.

Seine River Estuary, France. About 35 % of females exhibited swimming legs that had setae with slightly seen segmentation but no segments were found in caudal rami setae as in *E. carolleeae*. Genital double-somite ratio (W2/W1) in the Seine population (1.23 + 0.089) was also significantly less than in *E. carolleeae*. In *E. affinis* females in the Seine Estuary the tiny spine of leg 5 was significantly longer (0.24 + 0.025) than in *E. carolleeae* (see Tables 2, 4). All *E. affinis* males in the Seine Estuary had caudal rami with spines on the dorsal surface and a large outgrowth on the left basipod of P5.

Schelde River Estuary, Belgium. The tiny spine on P5 of *E. affinis* females in the Schelde had the maximum length among all populations studied (Table 4). In male P5 the left basipod L/W ratio also had a minimal value (see Table 4). This *E. affinis* population seems like more morphologically divergent from *E. carolleeae* than other European populations.

This overview lets us conclude that characters used here to discriminate *E. carolleeae* are sufficient to distinguish all geographically distant populations of *E. affinis* from the Northern part of the Gulf of Finland in the Baltic to the South of Spain (see Fig. 1). Only a low number of individuals from some populations of *E. affinis* showed a small overlap in certain, but not all, characters with *E. carolleeae*. In some cases (for example population in the Ust-Luga), it was caused by the *E. carolleeae* invasion that was confirmed by molecular-genetic tools (Alekseev *et al.* 2009).

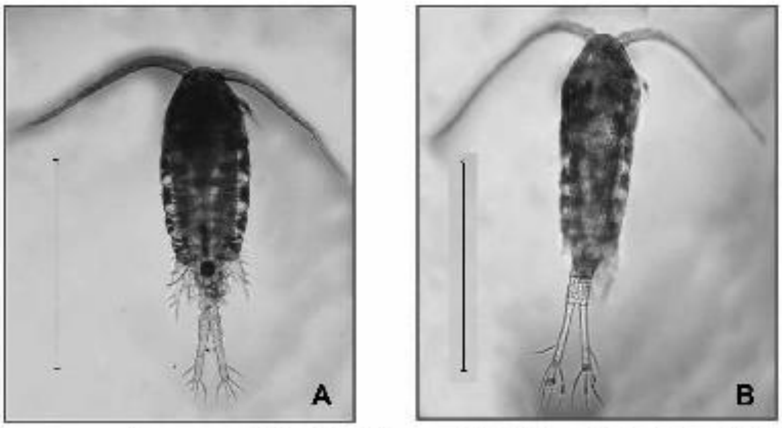


FIGURE 8. *Eurytemora carolleeae* **sp. nov.** (A) and *E. affinis* (Poppe, 1880) (B) from the Luga Bay, The Finish Gulf, Baltic Sea. (Photo: Mrs Natalia Sukhikh)

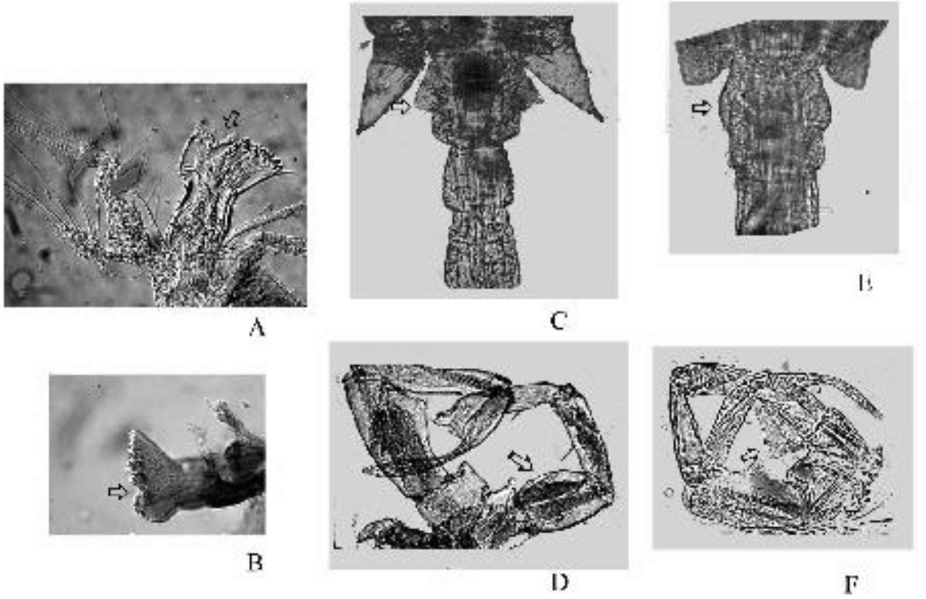


FIGURE 9. *Eurytemora carolleeae* **sp. nov.** (A–D) and *E. affinis* (Poppe, 1880) (E–F): A, female mandible, arrow indicating a gap; B, male mandible, arrow indicating a gap; C, female genital somite with wing-like outgrowth; D, male leg 5 with arrow indicating left basipod; F, female genital somite without wing-like outgrowth; F, male P5, arrow indicating left basipod. (Photo: Mrs Natalia Sukhikh)

Discussion

Lee and Frost (2002) combined the molecular genetic results of Lee (2000) with brief morphometric analysis of *Eurytemora cf. affinis* collected from 43 sites around the Holarctic. They had already proposed that the European populations are quite distinct from the North Atlantic clade and also from all the other clades of *E. affinis* (Poppe, 1880). Though they declared that there are morphological distances among the studied *E. affinis* populations, in fact they did not suggest any clear morphological differences between the North Atlantic and European clades. In their conclusion, they revealed morphological stasis in the *E. affinis* group and concluded that due to the long–term reproductive isolation and molecular evolution, the four major Asian, European, North Atlantic and Pacific clades became a complex sibling species "where speciation was accompanied by lack of morphological differentiation." Lee & Frost (2002) used for their analysis 3–5 individuals from each site. For morphometry, they measured a limited number of structures. Female characters included genital double-somite proportion, and ratio of the 22nd and 24th segments of the first antenna. Male characters were limited to some ratios in P5 and, as in the female, the ratio of the 22nd and 24th segments of the first antenna. These characters were selected by the authors because they are typically used for copepod taxonomy. We argue that in a sibling species complex this approach might not be an effective tool to distinguish closely related species.

That is why in our study we examined a larger amount of material. In the case of morphological variability up to 60 specimens from the type populations were measured. Additionally, before selecting morphometric indexes, we examined about 25 morphological structures in *E. affinis* from the type locality in the Elbe estuary and in specimens from the North Atlantic clade from the Chesapeake Bay. Then all the selected morphological differences were checked (in the case of morphometric indexes with appropriate statistic methods) in 8 other distant populations in these two species. This analysis let us come to some extent to the opposite conclusion than Lee & Frost (2002) on the possibility of separating and describing a new species in this complex based on external morphology.

Dodson *et al.* (2010) used measurements of 26 characters from 125 female specimens to determine patterns of morphological variation in *Eurytemora* species within the ancestral range in Alaska. The goal of their study was to analyze morphological variation and divergence within this genus, focusing on *Eurytemora* species that inhabit North America. As a result they excluded from consideration morphological characters of *E. affinis* from Europe and were not able to separate the North Atlantic clade from the native *E. affinis*.

One of the possible additional explanations why previous researchers did not find morphological differences between these two taxa could be the lack of material from the type population from the Elbe River in their analysis. Without a re-description or at least detailed analysis of the type population of a species described in the 19th century, to our mind, it is impossible to separate a new species in a sibling species complex.

On the level of molecular-genetic distances among clades studied by Lee (2000), it is also clear to us that so-called Asian and Pacific clades, which are not of course taxonomically valid names if based on genetic differentiation alone, very soon will appear as new species or at least subspecies. A preliminary morphological survey (Alekseev, unpublished) indicates that they can be discriminated from *E. affinis* and *E. carolleeae* **sp. nov.** These taxa, when described, will define the *E. affinis* complex more clearly based on morphological differentiation. This will be especially important if more new species of this complex cross geographical borders via human mediated vector.

Among the European populations studied here practically no significant variation was observed in the P5 characters both in males and females. In female *E. affinis* from the most studied European sites, the tiny spine in distal exopod segment P5 was about 20% and sometimes about 30% of the nearest small spine in length. In males, *E. affinis* length/width proportion in basipod left P5 was about 1, due to an asymmetrical outgrowth on the inner side of the leg they possess (see Table 4). These characters of P5 construction are recommended as the most valuable ones for morphological separation between these two taxa because, due to the recent invasions, they can be found together, both in Europe and in North America (Busch & Brenning 1992; Alekseev *et al.* 2009).

Compared to the other 16 congeners, *E. carolleeae* differs by a combination of characters that in females include: genital double-somite with lateral "wings" of different sizes, symmetrical P5 in the first exopod segment with smooth inner outgrowth, oriented under 45 degrees to the segment axes and with two sub-equal spines; coxae of legs 1–4 with long setae at inner side; more or less symmetrical abdomen; caudal rami and last abdominal segment covered with dense spines.

Males in *E. carolleeae* differ from other congeners by: a long seta at inner side coxa in legs 1–4; 4-segmented right leg in P5; caudal rami naked on both dorsal and ventral surfaces; last abdominal segment covered with rather strong dents; 8–12 segments of the first antenna armed with spines, the spine at 12th segment at least two times as long as other spines; in P5 left basis more or less cylindrical in shape, provided with two small round shape outgrowth, the first segment of left exopod without very long spine distally and the second (distal) segment with 2 lobes at the end.

Beside the Atlantic coast of USA *E. carolleeae* was also found in samples collected in Canada (The St. Lawrence Estuary, Dr. G. Winkler donation). Specimens from these two populations are morphologically close, but clearly different from *E. affinis* from the Elbe River population (Fig. 10). Analysis of published drawings and digital pictures of *E. cf. affinis* lets us conclude that there is at least one record of *E. carolleeae* in freshwater environments in Mexico from a reservoir in a desert area (Suárez-Morales *et al.* 2008).

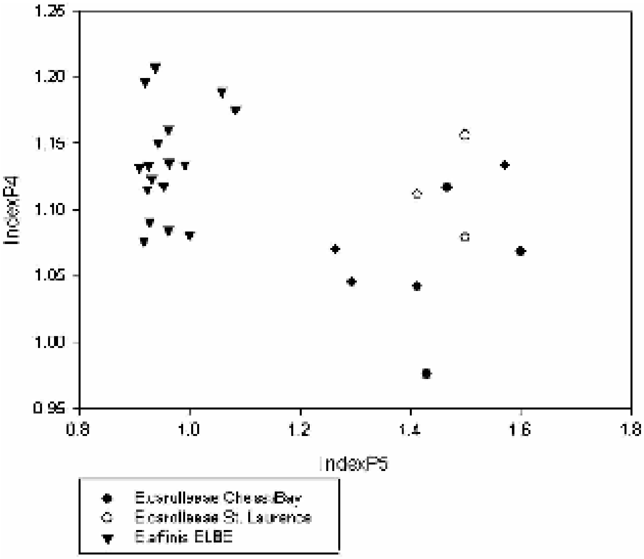


FIGURE 10. Morphological indexes in *Eurytemora affinis* (Poppe, 1880) from the type locality and *E. carolleeae* **sp.nov.** from the Chesapeake Bay, USA and St. Lawrence Bay, Canada. Males: IndexP4, distal spine/segment length ration in EXP P4; IndexP5, L/W ratio in BAS P5 left. For more explanation see text.

Specimens of the Atlantic clade of *E. affinis* from the Chesapeake Bay (= *E. carolleeae*) were recently found in the Gulf of Finland, Baltic Sea (Alekseev *et al.* 2009).

Eurytemora carolleeae shows a tendency to advance further inland in continental waters (Lee & Peterson 2003; Suárez-Morales *et al.* 2008). In the new invasive area in Europe its distributive range will possibly be significantly modified in the near future. On the other hand, all previous records of *E. affinis* in North America should now be checked again to avoid mixing the newly described *Eurytemora carolleeae* with *E. affinis* that recently invaded from Europe (Busch & Brenning 1992; Debiase & Taylor 1993; Suárez-Morales & Reid 1994, 1998; Suárez-Morales *et al.* 2008).

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