Universidade de Aveiro Departamento de Biologia
2012

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Interações ecológicas em populações sujeitas a *stress* ambiental

Ecological interactions in populations facing environmental stress

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Documento apresentado à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia realizada sob a orientação científica do Doutor Fernando José Mendes Gonçalves, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro, da Doutora Maria Arminda Pedrosa e Silva Carvalho, Professora Auxiliar da Faculdade de Ciências e Tecnologia da Universidade de Coimbra, e do Doutor Bruno Branco Castro, Investigador Auxiliar do Departamento de Biologia & CESAM, Universidade de Aveiro.

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Dedico este trabalho aos meus pais, à minha irmã e ao meu marido.

o júri

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palavras-chave

Stress ambiental, interação genótipo-ambiente, fatores de *stress* abióticos, interações bióticas, educação científica

resumo

O zooplâncton, particularmente os cladóceros, são organismos de água doce importantes na regulação da produção primária dos ecossistemas de água doce. No entanto, também podem adaptar-se a condições salobras. Tendo em conta as previsões no âmbito das alterações climáticas, a intrusão salina pode ocorrer a par com a subida de temperatura. As populações de água doce podem ficar vulneráveis aos efeitos interativos da salinidade e da temperatura, de acordo com os seus limites de tolerância e capacidade de adaptação ao *stress* ambiental. Assim, a presente tese analisou as interações resultantes das alterações destes agentes de *stress* em populações de cladóceros de água doce. Primeiro, comparou-se a halotolerância de diferentes genótipos de *Simocephalus vetulus* provenientes de populações de água doce e de água salobra de modo a avaliar a existência de uma componente genética de resistência à salinidade. A sensibilidade aguda dos genótipos variou na mesma gama de concentrações; todavia, todos os genótipos da população salobra, exceto um, foram mais tolerantes do que os de água doce, em termos de tempo à imobilização. Contudo, não foi possível estabelecer uma relação entre a performance reprodutiva em condições salobras e o contexto ambiental de origem destes genótipos. Mais, estes ensaios mostraram que as populações de água doce têm potencial para tolerar incrementos de salinidade. Como tal, pode-se concluir que a seleção a que os genótipos estão sujeitos no seu local de origem foi mais fraca do que o esperado. Segundo, investigou-se a capacidade de aclimatação de *Daphnia galeata* à salinidade e temperatura, de modo a avaliar a halotolerância de *Daphnia* a duas temperaturas num cenário de aclimatação multigeracional. O objetivo foi compreender se a pré-adaptação ao *stress* ambiental (20ºC e 25ºC *versus* 0 g/L e 1 g/L de NaCl) influenciou posteriormente as respostas a estes agentes de stress. Verificou-se uma tendência para um aumento de sensibilidade ao NaCl, a temperaturas mais elevadas. No entanto, este efeito foi anulado após nove gerações, mas apenas quando os organismos foram aclimatados aos dois agentes de *stress* em simultâneo (salinidade e temperatura elevada). Terceiro, demonstrou-se experimentalmente que a salinidade interferiu com a competição inter-específica, alterando a composição das comunidades zooplanctónicas.
Este conjunto de evidências permitiu-nos refletir nos múltiplos impactos de agentes de *stress*, particularmente os relacionados com as previsões de alterações climáticas. Em paralelo aos estudos de natureza experimental, e numa perspetiva de Educação para o Desenvolvimento Sustentável (EDS), importa também promover o desenvolvimento de competências necessárias à compreensão de mudanças ambientais globais (e.g., o impacto da salinidade e da temperatura) para implementar estratégias de mitigação e adaptação. Neste contexto, foi realizada uma atividade com estudantes do ensino secundário, que se tornou uma boa oportunidade para a sua aprendizagem e aquisição de competências de interpretação de dados experimentais, assim como de sensibilização para as questões ambientais.

keywords

Environmental stress, genotype-environment interaction, abiotic stressors, biotic interactions, scientific education

abstract

Zooplankton, namely cladocerans, are important regulators of primary production in freshwater ecosystems. However, these filter-feeders can also be found in brackish waters and can therefore adapt to this stressful condition. Bearing in mind climate change predictions, saline intrusion can occur in coastal freshwater systems, accompanied by increasing temperatures. Freshwater populations can thus become vulnerable to the interactive effect of salinity and temperature, framed within their tolerance limits and adaptation ability to environmental stress. Taking this into consideration, the present thesis aimed at addressing the complex interactions resulting from the challenge of these environmental stressors on freshwater cladoceran populations. First, the halotolerance of different *Simocephalus vetulus* genotypes from two freshwater and one brackish population (salinity 2.3) was explored. The goal was to determine whether there was a genetic component of salinity resistance. Clone acute sensitivity was comparable among populations. However, all brackish clones, except one, were more halotolerant than freshwater genotypes, in terms of survival time. Life history assays, carried out with extreme genotypes (from previous experiment) revealed no relationship between fitness and the original environmental context of genotypes. Also, these experiments showed that freshwater populations do have the potential to tolerate salinity increases. As such, we can conclude that the local selective pressure caused by salinity was weaker than expected. Second, the acclimation ability of *Daphnia galeata* to salinity and temperature was investigated in this study, by evaluating *Daphnia*'s sensitivity to salinity, at two different temperatures, along a multigenerational acclimation scenario. The objective was to understand whether pre-adaptation to environmental stress (20ºC and 25ºC versus 0 g/L and 1 g/L of added NaCl) influenced the response to the latter exposure to these stressors. A tendency towards the increase of sensitivity to NaCl was observed, at higher temperature. However, this temperature-dependent effect was nullified after nine generations, but only when animals had been acclimated to both stressors (salinity and higher temperature). Third, we experimentally demonstrated that salinity interfered with inter-specific competition, altering the community composition of zooplankton communities. This body of evidence allowed us to reflect on the impacts of multiple stressors, particularly those related to climate change predictions. In parallel with experimental studies, within the framework of Education for Sustainable Development, it is also important to promote the development of the necessary competences towards the comprehension of global environmental change (e.g. the impact of salinity and temperature), in order to implement adequate mitigation and adaptation strategies. Hence, an activity with secondary school students was conducted, providing a good opportunity towards learning and competence acquisition in the interpretation of experimental data, as well as increasing awareness towards environmental issues.

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Introdução Geral

ENQUADRAMENTO DA TESE

Stress ambiental como agente de seleção

As relações estruturais, funcionais e de produtividade das comunidades bióticas são reguladas, entre outras, pelas condições ambientais (Ricklefs, 2003). Estas englobam fatores físicos e químicos, variáveis no tempo e no espaço, e constituem o *habitat* dessas comunidades (Ricklefs, 2003; Castro, 2007). As relações tróficas que os organismos num dado local estabelecem entre si, e com as condições ambientais locais, moldam a estrutura e dinâmica funcional dos ecossistemas (Lampert & Sommer, 1997; Ricklefs, 2003). Tendo em consideração as relações tróficas nas comunidades bióticas, é de assinalar a transferência de energia e transformação de matéria ao longo das cadeias alimentares, que se mantêm em equilíbrio dinâmico (Lampert & Sommer, 1997; Ricklefs, 2003). No entanto, o "equilíbrio dinâmico" dos ecossistemas é relativo ao saldo mais ou menos constante de energia no sistema, sem que isso signifique constância das condições ambientais (condicionantes abióticas e bióticas); aliás, o cenário real é exatamente o oposto.

Os ecossistemas estão sujeitos a constantes flutuações do meio, de natureza esporádica ou regular (ritmos diários ou sazonais). Estas flutuações podem ser devidas a agentes de *stress* abióticos ou a agentes de *stress* bióticos (Bijlsma & Loeschcke, 2005). Além disso, podem ainda ser causadas pela interação entre estes agentes de *stress,* estando, portanto, o seu equilíbrio dinâmico dependente da interação entre fatores abióticos e interações bióticas (Ricklefs, 2003; Castro, 2007). As flutuações a que as populações estão sujeitas resultam do designado *stress* ambiental, o qual pode ser caracterizado como uma força modeladora de evolução em ambientes em mudança (Bijlsma & Loeschcke, 2005). O *stress* ambiental obriga as populações a uma constante resposta fenotípica e genotípica, envolvendo mecanismos adaptativos para reduzir o impacto negativo desse mesmo *stress*. O produto da interação das comunidades bióticas com os agentes de *stress* bióticos e abióticos traduz-se na adaptação, atuando ao nível dos fenótipos da população, resultantes de respostas a fatores ambientais (Stearns, 1992; Ricklefs, 2003). As populações naturais podem sofrer modificações adaptativas das expressões fenotípicas, num dado momento perante pressões seletivas abióticas ou bióticas, assim como da interação entre ambas (De Meester, 1997; Cousyn et al., 2001; Hart & Bychek, 2011). Por inerência, as populações apresentam a capacidade de se adaptar a alterações das condições ambientais, da disponibilidade de recursos ou das interações bióticas (Ricklefs, 2003). A modificação do seu comportamento, do tamanho corporal e das suas respostas reprodutivas são exemplos dessa capacidade adaptativa, de modo a aumentar o seu sucesso perante modificações no equilíbrio do seu *habitat* (Hart & Bychek, 2011). Quando as perturbações dos ecossistemas são bruscas e esporádicas, as populações não têm tempo para se adaptarem e apenas sobrevivem os organismos mais aptos dessas populações. No entanto, estes fenótipos são a expressão dos genótipos correspondentes e que constituem o *pool* genético da população (Lampert & Sommer, 1997). Perante a variabilidade fenotípica e genotípica de uma população, os indivíduos com maior aptidão e maior taxa de sobrevivência são favorecidos, otimizando o potencial reprodutivo da população (Lampert & Sommer, 1997; Hart & Bychek, 2011). Deste modo, as populações presentes num dado ecossistema, num dado momento, possuem as características necessárias para ali existir, resultado da pressão exercida pelas condicionantes abióticas e bióticas locais sobre os fenótipos dos indivíduos e o *pool* genético da população.

Os fatores abióticos, como luz, temperatura, salinidade, oxigénio, pressão e disponibilidade de nutrientes exercem pressões seletivas sobre as populações aquáticas, nomeadamente ao nível do tamanho corporal dos organismos (Hart & Bychek, 2011). A predação e a competição apresentam-se igualmente como pressões seletivas bióticas sobre as populações e determinam o tamanho corporal dos organismos (De Meester, 1997; Boersma et al., 1999; Cousyn et al., 2001; Hart & Bychek, 2011). O tamanho corporal é inequivocamente reconhecido como uma importante característica da biologia funcional e ecologia animal, pois controla funções fisiológicas fundamentais, tais como a taxa metabólica, a taxa de filtração, as taxas de crescimento e a fecundidade das populações (Hart & Bychek, 2011), determinando a capacidade competitiva e a *performance* demográfica das espécies (Hart & Bychek, 2011), o que terá implicações na abundância de indivíduos na população e na definição da estrutura das comunidades nos ecossistemas (Hart & Bychek, 2011). No entanto, o investimento no tamanho corporal depende da disponibilidade de alimento e da consequente alocação de energia para os processos metabólicos fundamentais (Hart & Bychek, 2011). Perante condições ambientais desfavoráveis, estes organismos tendem a alocar energia para a reprodução e só depois a investir no crescimento (Smolders et al., 2005; Pereira, 2008). As alterações na disponibilidade de recursos alimentares são detetadas pelas populações, que utilizam essa informação para ajustar o investimento de energia nas estratégias reprodutivas (Gliwicz & Guisande, 1992; Hart & Bychek, 2011).

O contexto ambiental na dinâmica das populações aquáticas

As condições ambientais moldam o comportamento e o sucesso reprodutivo dos organismos que habitam um determinado local, uma vez que estes se adaptam às condições ambientais a que estão sujeitos com um intervalo de tolerância suficiente para o seu desenvolvimento (Ricklefs, 2003). Consequentemente, mudanças nas condições ambientais causam *stress* ambiental e podem comprometer a dinâmica das populações (Ricklefs, 2003). As populações de zooplâncton presentes em *habitats* lênticos de água doce são um grupo dominante nos ecossistemas aquáticos, quer em termos de número, quer em termos funcionais (Sarma et al., 2005; De Meester et al., 2006; Sarma & Nandini, 2006; Hart & Bychek, 2011), e um dos mais bem adaptados a viver em condições ambientais flutuantes (ver abaixo). Assim, estas populações apresentam uma dinâmica particular e extremamente importante para o equilíbrio dos ecossistemas em que se inserem, tal como referido anteriormente.

A dinâmica populacional do zooplâncton é sazonal, tal como é descrita pelo modelo sazonal do plâncton (PEG – Plankton Ecology Group) (Sommer et al., 1986). Segundo o modelo PEG, as baixas temperaturas e o curto período diurno no inverno levam a um reduzido crescimento fitoplanctónico, o que se traduz numa acumulação de nutrientes e numa diminuição da disponibilidade de alimento para os zooplanctontes, nomeadamente cladóceros de maiores dimensões. Em consequência, estes organismos diminuem a sua fecundidade e muitos recorrem a estratégias de diapausa, produzindo estruturas de dormência (Hairston, 1996; Castro, 2007). À medida que o inverno chega ao fim, o aumento da intensidade luminosa e a disponibilidade de nutrientes permitem o crescimento ilimitado de fitoplâncton (microalgas autotróficas), iniciando-se uma fase muito produtiva para o plâncton. Esta fase inclui o desenvolvimento de pequenas algas de crescimento rápido, as quais são alvo da herbivoria muito eficiente de espécies zooplanctónicas de crescimento mais lento e maior biomassa, nomeadamente cladóceros pertencentes aos géneros *Daphnia* e *Simocephalus* (Asaeda & Acharya, 2000). Assim, a biomassa algal mantém-se a níveis muito baixos, devido ao aumento do número de indivíduos filtradores, o que leva ao aumento das taxas de filtração de fitoplâncton, permitindo manter a água com reduzida turbidez (Jeppesen et al., 1999; Scheffer, 1999; Hairston et al., 2005; Castro, 2007). Os nutrientes são reciclados pela herbivoria, tornando-se novamente disponíveis para os produtores (fitoplâncton). O controlo do crescimento algal permite manter o estado de transparência da água (Jeppesen et al., 1994; Hairston et al., 2005; Jeppesen et al., 2007b), que é essencial para evitar a eutrofização dos ecossistemas de água doce (Scheffer, 1999; Castro, 2007). É de destacar o papel vital dos organismos da família Daphniidae (particularmente *Daphnia*) enquanto herbívoros reguladores da produtividade fitoplanctónica (Brooks & Dodson, 1965; Gliwicz, 1990; Scheffer, 1999), na medida em que os zooplanctontes de pequenas dimensões não são capazes de controlar eficientemente o crescimento algal (Brooks & Dodson, 1965; Declerck et al., 1997; Tessier et al., 2001).

Com o avanço do verão, há um aumento progressivo da pressão predatória (por parte dos peixes) sobre os cladóceros de grandes dimensões, o que mantém a sua abundância baixa. Com a ausência de competição entre indivíduos de diferentes tamanhos, a comunidade zooplanctónica é dominada por cladóceros de pequenas dimensões, menos eficientes no controlo da biomassa algal (Castro, 2007). Em consequência, desenvolve-se novamente a comunidade fitoplanctónica, o que reduz fortemente a transparência da água. O verão corresponde ao período mais vulnerável dos sistemas aquáticos, na medida em que temperaturas elevadas e elevada disponibilidade de nutrientes (recorrentemente por ação humana) podem provocar a diminuição da qualidade da água (Wetzel, 1993; Boavida, 2000), com a ocorrência de episódios de sobrecrescimento algal (e episódios de anoxia) ou a dominância de cianobactérias - microorganismos potencialmente nocivos (Boavida, 2000; de Figueiredo et al., 2004; Vasconcelos, 2006). Com a chegada do outono restabelece-se a quantidade de nutrientes necessários ao aumento da produção fitoplanctónica, podendo ressurgir cladóceros de grandes dimensões (Sommer et al., 1986; Castro, 2007). A dinâmica populacional do plâncton está dependente do grau de plasticidade e adaptabilidade destes organismos às constantes modificações no seu *habitat*, quer de fatores abióticos (*e.g.* salinidade, temperatura, disponibilidade de nutrientes), quer de interações bióticas (*e.g.* predação, competição, parasitismo, herbivoria) (Brönmark & Hansson, 1998; Castro, 2007).

Stress ambiental e alterações climáticas: salinização secundária

Os ecossistemas aquáticos de água doce podem sofrer perturbações graves, nomeadamente ao nível das lagoas costeiras. Em Portugal, existem lagoas naturais (ou semi-naturais) costeiras, cuja origem coincide com a formação do cordão dunar em zonas de aquíferos muito superficiais [*e.g.* sistema de lagoas entre Mira e a Figueira da Foz (Gonçalves et al., 1996; Castro et al., 2005), lagoas a norte de Sines (Freitas et al., 2002; Cabral et al., 2006)], e que constituem raros e valiosos *habitats* de água doce. Dada a sua localização, estes *habitats* são extremamente vulneráveis devido às pressões antrópicas e a fatores associados às alterações climáticas (*e.g.* temperatura, salinidade, eventos extremos). As alterações climáticas são um problema global atual cujas consequências podem ocorrer a vários níveis, como descrito em documentos internacionais (IPCC, 2002, 2008). A salinização de lagoas costeiras de água doce é uma das consequências das alterações climáticas (Schallenberg et al., 2003), podendo ocorrer de forma intensiva ou num cenário progressivo, com incrementos suaves de salinidade (Schallenberg et al., 2003; Nielsen et al., 2008; Loureiro et al., 2012). Esse processo de salinização pode ocorrer esporadicamente, por entrada de água do mar em lagoas de água doce promovida por uma conexão intermitente com o mar (Loureiro et al., 2012), ou devido a variações do nível médio da água do mar em consequência das alterações climáticas (Hall & Burns, 2002; Schallenberg et al., 2003). Por outro lado, a salinização de lagoas de água doce pode também ocorrer por via subterrânea, devido a secas prolongadas (Marques et al., 2007; Nielsen & Brock, 2009) e sobreexploração de aquíferos (Lambrakis, 2006), num processo denominado intrusão salina (IPCC, 2002, 2008). Consequentemente, as espécies sujeitas a aumentos de salinidade tornam-se vulneráveis e o equilíbrio dos mecanismos ecológicos fica dependente da tolerância das espécies à salinidade (Arnér & Koivisto, 1993; IPCC, 2002; Nielsen et al., 2003; Schallenberg et al., 2003; IPCC, 2008).

A perturbação dos organismos devido à salinização tem consequências na sua distribuição, sobrevivência, crescimento e reprodução (Hall & Burns, 2002; Smolders et al., 2005; Gonçalves et al., 2007; Brucet et al., 2010; Duchet et al., 2010), afetando a dinâmica e a estrutura genética das populações (Schallenberg et al., 2003; Sarma et al., 2006; Santangelo et al., 2008). Em sistemas aquáticos de água doce, a salinidade é um agente de *stress* natural que exerce pressões seletivas locais sobre os diferentes genótipos de cada população, afetando diretamente a estrutura das populações (Sarma et al., 2006;

Jeppesen et al., 2007b). A resposta das populações de dafniídeos (cujo papel ecológico foi destacado em cima) sujeitas a alterações das condições ambientais (*e.g.* salinidade) está dependente da variabilidade dentro e entre as populações (devido à plasticidade fenotípica e aos polimorfismos genéticos - De Meester, 1997; Boersma et al., 1999; Cousyn et al., 2001; De Meester et al., 2006). Os dafniídeos são característicos de água doce (Alonso, 1996; Sarma et al., 2006) e parecem ser sensíveis a pequenos incrementos de salinidade (Hall & Burns, 2002; Schallenberg et al., 2003; Gonçalves et al., 2007). No entanto, também podem ser encontrados em água salobra (Teschner, 1995). A tolerância à salinidade depende por isso da capacidade de osmorregulação dos genótipos da população de dafniídeos (Aladin & Potts, 1995; Jeppesen et al., 2007b).

A presença de um agente de *stress* pode produzir fenómenos adaptativos e existem evidências de que a salinidade pode funcionar como agente de seleção em espécies dulçaquícolas (ver abaixo). A variabilidade genotípica causa diferenças nas respostas a determinadas alterações ambientais, como por exemplo a intrusão salina (esporádica ou contínua) em meios de água doce, favorecendo populações halotolerantes (Teschner, 1995). Estas populações possuem características genéticas que favorecem a sua capacidade de sobrevivência e reprodução no seu ambiente de origem, o que pode levar ao fenómeno de adaptação local (De Meester, 1996a, 1996b; Boersma et al., 1999). Alguns estudos demonstraram que existem diferenças na estrutura genética de populações de dafniídeos de diferentes *habitats* (Weider & Hebert, 1987; Teschner, 1995). Se esta diferenciação genética resulta numa resposta adaptativa a forças seletivas locais, então os organismos devem ter melhor desempenho reprodutivo no seu local de origem do que noutros locais (Teschner, 1995; De Meester, 1996a, 1996b; Boersma et al., 1999). A adaptação local ocorre se existirem diferenças genéticas inter-populacionais significativas para determinadas características (Boersma et al., 1999). A adaptação local nas populações de dafniídeos tem-se focado no efeito da pressão predatória (*e.g.* De Meester, 1996a, 1996b; Boersma et al., 1999; De Meester et al., 2002; Declerck & Weber, 2003). No entanto, outros fatores (*e.g.* salinidade e temperatura) podem levar à ocorrência de diferenças significativas na estrutura genética das populações e causar fenómenos microevolutivos (Teschner, 1995; Van Doorslaer et al., 2007; Van Doorslaer et al., 2010). A diferenciação genética local em termos de halotolerância já foi demonstrada em algumas populações de dafniídeos de ambientes salobros (Weider & Hebert, 1987; Teschner, 1995).

Ainda em populações dulçaquícolas, torna-se importante o estudo do impacto da salinidade conjugado com outros fatores também associados às alterações climáticas, como por exemplo, a temperatura. O possível aumento de temperatura influencia também a dinâmica trófica do zooplâncton (Brucet et al., 2010; Van Doorslaer et al., 2010). Nestas condições, a pressão predatória de peixes sobre o zooplâncton de maiores dimensões aumenta, prevalecendo o zooplâncton de pequenas dimensões, o que acarreta a diminuição da capacidade global de filtração da comunidade zooplanctónica (Jeppesen et al., 2007a; Brucet et al., 2010). Daqui resultam impactos importantes na transparência e qualidade da água (ver dinâmica do plâncton, acima).

Modelos biológicos para o estudo de *stress* ambiental nas populações aquáticas

As comunidades zooplanctónicas de ecossistemas de água doce, particularmente os dafniídeos (Cladocera, Daphniidae), tornaram-se um grupo central no estudo da ecologia aquática de lagos e lagoas (Lampert, 2006). Este grupo de organismos tem sido estudado como modelos fisiológicos, como herbívoros modelo e como presas modelo (Lampert, 2006). A vantagem da utilização destes organismos reside num conjunto de características que estes modelos biológicos possuem, nomeadamente a nível ecológico, fisiológico e logístico.

Do ponto de vista ecológico, as diversas espécies de dafniídeos têm um papel central no equilíbrio ecológico nas teias alimentares dos ecossistemas de água doce (Wetzel, 1993) e possuem uma dinâmica populacional bem conhecida (Sommer et al., 1986). Estes organismos são essencialmente herbívoros e filtradores (Alonso, 1996; Lampert, 2006), no entanto também se alimentam de bactérias e protozoários (Lampert, 2006). Este tipo de alimentação torna os dafniídeos, principalmente os de maiores dimensões (Brooks & Dodson, 1965; Asaeda & Acharya, 2000), importantes reguladores da produção primária, reciclagem de nutrientes e transferência de energia para os níveis tróficos superiores (Hall & Burns, 2002; Sarma et al., 2005; Sarma et al., 2006; Gonçalves et al., 2007). Esta função reguladora resulta do facto destes dafniídeos constituírem a ligação entre os produtores (algas) e os consumidores de níveis tróficos superiores (peixes) (Sarma et al., 2005; Sarma et al., 2006), ocupando uma posição chave na teia trófica pelágica (Brönmark & Hansson, 1998; Castro, 2007). Como tal, os dafniídeos são indicadores do estado trófico de um lago (Wetzel, 1993; Caramujo & Boavida, 2000) e da qualidade da água (Colomer, 1996). O crescimento populacional destes organismos é controlado simultaneamente pelas flutuações na disponibilidade de alimento (McCauley et al., 2008) e pela ação predatória dos peixes (Jeppesen et al., 1997; Brönmark & Hansson, 1998; Castro, 2007).

Do ponto de vista fisiológico, estes organismos apresentam um ciclo de vida curto, reprodução predominantemente partenogenética e produzem ninhadas com elevado número de indivíduos. Possuem dimensões corporais reduzidas, apresentam elevadas taxas de crescimento populacional e são sensíveis a uma grande variedade de substâncias tóxicas (Lampert, 2006; Sarma & Nandini, 2006; Marques et al., 2011). No meio natural, os dafniídeos (e os cladóceros, em geral) apresentam dois tipos de reprodução (partenogénese cíclica): sexuada e assexuada (Wetzel, 1993). A reprodução sexuada ocorre apenas quando as condições do meio são desfavoráveis (sobrepopulação, indisponibilidade de alimento). Nestas condições, as fêmeas produzem um macho que as fecunda e produzem ovos de resistência encapsulados numa estrutura denominada *ephippium* (Davison, 1969; Hairston, 1996). Os ovos de resistência depositam-se no sedimento, onde se mantêm em estado de dormência até que as condições ambientais sejam favoráveis à sua eclosão. A reprodução sexuada introduz variabilidade genética nas populações destes organismos, o que em termos evolutivos é bastante importante (Hairston, 1996). Assim, quando ocorre a eclosão dos ovos de resistência, a população apresenta uma grande variabilidade de genótipos e maior possibilidade de sobrevivência a modificações das condições do meio (Brucet et al., 2010). Quando as condições ambientais são favoráveis, os dafniídeos reproduzem-se assexuadamente por partenogénese cíclica. Nestas condições, cada fêmea dá origem a um elevado número de descendentes fêmeas. Com este tipo de reprodução, os dafniídeos podem aumentar rapidamente o seu número numa lagoa de água doce. A fisiologia e desenvolvimento dos dafniídeos são afetados, quer por interações bióticas, quer por fatores abióticos, o que tem consequências para os restantes componentes da teia alimentar pelágica (Lampert, 2006). Assim, o seu papel como um modelo fisiológico é essencial para a compreensão da sua função como um modelo ecológico (Lampert, 2006), uma vez que mudanças ambientais que interferem com as suas características fisiológicas levam à ocorrência de perturbações na dinâmica populacional destes dafniídeos, afetando o equilíbrio das teias tróficas, a composição das comunidades e o funcionamento destes ecossistemas aquáticos (Wetzel, 1993; Altermatt et al., 2009).

Estes modelos biológicos permitem também desenvolver investigação sobre questões evolutivas gerais e avaliações ecotoxicológicas (De Meester, 1997; Lampert, 2006; Sarma & Nandini, 2006), permitindo compreender processos evolutivos devido a interações bióticas e abióticas em *habitats* dulçaquícolas, assim como avaliar a qualidade desse compartimento aquático (Sarma & Nandini, 2006; Sarma et al., 2006; Jeppesen et al., 2007b). Esta investigação pode ser desenvolvida no campo ou em laboratório. Os dafniídeos são vantajosos devido à fácil manipulação em laboratório (requerem volumes pequenos de meio de cultura) e disponibilidade de técnicas de cultura. Mais, devido à capacidade de manter populações laboratoriais permanentemente em reprodução partenogenética, genótipos de populações diferentes podem ser mantidos em cultura nas mesmas condições, amenizando o efeito das condições ambientais de onde provieram, e permitindo quantificar as diferenças inter-populacionais (De Meester, 1996a, 1996b; Boersma et al., 1999). A investigação ecológica e ecotoxicológica em populações de dafniídeos é muito mais intensa para o género *Daphnia*, existindo menos contribuições de literatura científica para outros géneros de dafniídeos, como *Simocephalus* e outros cladóceros (Sarma & Nandini, 2006). Os indivíduos do género *Daphnia* habitam a coluna de água e têm maior biomassa (ver modelo PEG), o que os torna filtradores muito eficientes, com maior impacto na biomassa algal e na transparência da água (Scheffer, 1999; Castro, 2007). A biomassa destes dafniídeos torna-os numa fácil presa visual para os predadores planctívoros, os quais exercem elevada pressão predatória sobre eles (Boersma et al., 1998; Lampert, 2006). Os indivíduos do género *Simocephalus* ocupam um nicho ecológico diferente de *Daphnia*, devido à sua preferência por *habitats* estruturados, nomeadamente com macrófitas (Alonso, 1996; Vakkilainen et al., 2004; Van Doorslaer et al., 2007). Os organismos deste género também apresentam taxas de filtração elevadas (Brito et al., 2006), podendo contribuir significativamente para o controlo do fitoplâncton na zona litoral de lagos e albufeiras (Scheffer, 1998). Comparativamente com *Daphnia*, *Simocephalus* poderá estar mais protegido em relação à predação devido ao seu nicho ecológico (Burks et al., 2002). Além disso, é sensível a determinados contaminantes (Martínez-Jerónimo & Martínez-Jerónimo, 2007; Martínez-Jerónimo et al., 2008), podendo apresentar potencial para estudos de ecotoxicologia. Assim, *Simocephalus* poderá constituir um bom modelo na avaliação da qualidade ambiental.

Construção de conhecimento e ensino das ciências

O estudo e compreensão de conceitos estruturantes em Biologia, tais como os referidos anteriormente (*e.g.* biodiversidade; dinâmica de ecossistemas; ecossistemas aquáticos; cadeias alimentares), e o conhecimento construído nos capítulos seguintes, são relevantes para alertar os cidadãos, particularmente alunos, para as problemáticas ambientais, tais como alterações climáticas, e suas consequências no funcionamento dos ecossistemas. Os alunos devem construir conhecimentos adequados sobre estes assuntos, uma vez que representam as gerações futuras e deverão saber tomar decisões de forma crítica e consciente (Boyes et al., 1993; Galvão & Freire, 2004; UN, 2005). No entanto, como problemas ambientais nem sempre são adequadamente compreendidos pelos alunos, a escola assume um papel fundamental no seu esclarecimento (Boyes et al., 1993). Assim, é importante que no ensino das ciências os professores promovam atividades de aprendizagem, procurando desenvolver competências, incluindo científicas, relevantes para eles próprios e para os alunos (McNeill & Krajcik, 2008). Alguns conteúdos abordados em trabalhos de investigação, como neste estudo (*e.g.* dinâmica de ecossistemas; equilíbrio de ecossistemas aquáticos; cadeias alimentares; agentes de *stress* bióticos e abióticos), permitem explorar ideias estruturantes identificáveis, numa perspetiva de Educação para o Desenvolvimento Sustentável (EDS), em temáticas que fazem parte do Currículo Nacional do Ensino Básico (CNEB) na área das Ciências Físicas e Naturais, como "Sustentabilidade na Terra", estando igualmente subjacente a objetivos inerentes à recém-proclamada Década das Nações Unidas para a Biodiversidade (2011-2020).

Assim, a identificação de possíveis articulações entre documentos curriculares e fontes credíveis de informação científica ajuda a promover a cooperação entre comunidades escolares e comunidades científicas responsáveis pela construção de conhecimento científico (Loureiro et al., 2008), uma vez que permitirá a construção e utilização de recursos educativos importantes para promover aprendizagens significativas (Mintzes et al., 2000; Novak, 2000). Além disso, através da construção de um currículo científico relevante (Fensham, 2000), facilita-se o desenvolvimento de consciência ambiental e ética sobre problemas quotidianos e promovem-se tomadas de decisão conscientes e fundamentadas por alunos e professores, como se preconiza em EDS (UN, 2005; Tilbury, 2011). Consequentemente, estimula-se o desenvolvimento de competências, nomeadamente competências científicas, que radiquem em princípios de sustentabilidade e valorização de literacia científica (OECD, 2009; Tilbury, 2011), o que é fundamental nos atuais contextos social, económico e ambiental (Loureiro et al., 2008; Pedrosa & Loureiro, 2008). O ensino das ciências deve, pois, orientar-se para o desenvolvimento de competências pelos alunos, promovendo o seu envolvimento em atividades promotoras das suas próprias aprendizagens (Mintzes et al., 2000; Aikenhead, 2009), de modo que se tornem capazes de construir conhecimentos e desenvolver competências, nomeadamente científicas, necessárias à implementação de estratégias de resolução ou mitigação de problemas (Mintzes et al., 2000; OECD, 2009; Vieira et al., 2011).

Os problemas abordados devem surgir de contextos quotidianos, em que os professores devem implementar o currículo numa lógica de educação científica orientada para a resolução de problemas, através de trabalho de pesquisa de informação, valorizando-se ligações inter e transdisciplinares e desenvolvendo competências, atitudes e valores relevantes do ponto de vista social e pessoal (Martins, 2002). Esses problemas podem, por exemplo, estar relacionados com problemas globais atuais, como as alterações climáticas e as suas consequências. Como tal, deve-se orientar os currículos de ciências segundo uma perspetiva de Ciência-Tecnologia-Sociedade (CTS), para minimizar o desfasamento entre os currículos e os contextos reais (Martins, 2002; Aikenhead, 2009; Vieira et al., 2011). O desenvolvimento científico e tecnológico veio trazer mudanças na educação em ciências (Vieira et al., 2011), devendo-se promover um ensino com uma orientação CTS e promotor de pensamento crítico, que forme cidadãos cientificamente literatos e conscientes na tomada de decisões (Magalhães & Tenreiro-Vieira, 2006; Aikenhead, 2009; Vieira et al., 2011).

Assim, o ensino das ciências pode aproximar os jovens das ciências e tecnologias se o for mais aliciante e abordar questões atuais, pois pode ser encarado como estando ao serviço da melhoria da qualidade de vida, principalmente de grupos mais desfavorecidos. Além disso, o ensino das ciências pode ser um veículo para o desenvolvimento e pode ter um papel social, nomeadamente na redução da pobreza e em práticas de desenvolvimento sustentável das gerações futuras (Boyes et al., 1993; Martins, 2002; Vieira et al., 2011). No entanto, esta orientação pode ter constrangimentos de implementação devido à diversidade de significados CTS e à organização do sistema de ensino (Martins, 2002; Aikenhead, 2009), uma vez que os programas escolares são extensos e complexos, podendo comprometer as aprendizagens e afetar a motivação dos alunos. Importa, portanto, selecionar temas educacionalmente relevantes que levem os jovens a alcançar saberes importantes para a sua formação (Aikenhead, 2009). Assim, a realização de atividades com alunos, apoiados por investigadores, pode ser relevante para aquisição de competências por alunos e professores, o que requer cooperação entre Universidades e escolas e é essencial numa perspetiva de EDS (UN, 2005; OECD, 2009; Tilbury, 2011).

OBJETIVOS E ESTRUTURA DA TESE

Tendo em conta as previsões de alterações climáticas e suas consequências para os ecossistemas, existe uma crescente preocupação ambiental no que concerne aos impactos destas mudanças sobre os seres vivos, nomeadamente ao nível da biodiversidade e do funcionamento dos ecossistemas. Considerando que a estrutura e dinâmica funcional dos ecossistemas estão dependentes das relações tróficas entre os organismos e das condições ambientais, alterações dessas condições podem tornar vulnerável o equilíbrio dos ecossistemas. Assim, este trabalho pretendeu contribuir para a compreensão da forma como populações aquáticas de água doce, em particular populações de cladóceros, respondem a agentes de *stress* abióticos (salinidade e temperatura) e bióticos (competição), abrangendo também a sua interação, tendo em consideração um cenário de intrusão salina em lagoas costeiras de água doce e aumento de temperatura global. O presente documento versou sobretudo sobre os impactos da salinização das águas doces, com cenários complexos e ambientalmente relevantes, que incluíram a incorporação da componente ambiente-genótipo na avaliação do impacto da salinização de massas de água doce (capítulos I e II), a interação da salinidade com a temperatura em cenários com e sem aclimatação (i.e. adaptação) prévia dos organismos (capítulo III) e a influência da salinidade na competição interespecífica (capítulo IV). No âmbito do presente trabalho, foi ainda feito um esforço no sentido de transpor as problemáticas aqui abordadas para o ensino das ciências (capítulo V).

O presente documento encontra-se dividido em cinco capítulos, cujos objetivos específicos foram:

- Compreender as interações ambiente-genótipo, comparando a halotolerância aguda de diferentes genótipos de populações de *Simocephalus vetulus*, provenientes de lagoas de água doce e de água salobra, procurando perceber se a componente genética destas populações tem influência na sua tolerância à salinidade (CAPÍTULO I);

- Avaliar a tolerância crónica a NaCl de genótipos selecionados de populações de *Simocephalus vetulus*, com base na sua halotolerância aguda previamente determinada, de modo a compreender se a componente genética e o local de origem têm efeitos na tolerância à salinidade em termos de desempenho reprodutivo (CAPÍTULO II);
- Compreender o efeito da interação entre salinidade e temperatura, incluindo a pré-adaptação prévia a estes agentes de *stress*, em populações de *Daphnia galeata*, procurando perceber se a interação destes agentes provoca alterações na sobrevivência e no crescimento dos organismos (CAPÍTULO III);
- Avaliar o efeito da salinidade no desfecho competitivo entre *D. galeata* e *S. vetulus*, tendo em conta o estudo prévio da sua tolerância à salinidade e a ordem de introdução das espécies no sistema experimental, procurando compreender potenciais consequências para estas populações num cenário de salinização secundária (CAPÍTULO IV);
- Demonstrar a influência da temperatura na tolerância à salinidade de duas espécies de *Daphnia*, através do desenvolvimento de trabalho prático com alunos do ensino secundário, como meio para promover aprendizagens significativas e contribuir para estimular o desenvolvimento de competências necessárias para utilizar conhecimento científico e tecnológico em resolução de problemas (CAPÍTULO V).

Tal como especificado nos objetivos, o presente documento tem uma estrutura dividida em capítulos, apresentando, cada um, objetivos próprios bem definidos, mas integrados no âmbito global do trabalho de investigação. Cada capítulo possui as suas próprias secções de Introdução, Material e Métodos, Resultados, Discussão e Referências. Esta apresentação tem um formato mais próximo da publicação, pois cada um dos capítulos corresponde a um artigo submetido ou aceite para publicação em revistas de arbitragem científica internacional ou nacional. Por este motivo, alguns capítulos estão escritos em língua inglesa. O corpo da tese é precedido pela presente Introdução Geral, onde é feito o enquadramento da dissertação e são apresentados os seus objetivos. A última secção da tese consiste nas Considerações Finais, onde se procura refletir sobre a relevância do trabalho de investigação.

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Capítulo I

Genetic variability in the tolerance of natural populations of *Simocephalus vetulus* (Müller, 1776) to lethal levels of sodium chloride

O presente capítulo constitui um manuscrito publicado em revista internacional com arbitragem científica "Annales de Limnologie – International Journal of Limnology" e é, por esse motivo, apresentado na língua inglesa.

Genetic variability in the tolerance of natural populations of *Simocephalus vetulus* (Müller, 1776) to lethal levels of sodium chloride

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ABSTRACT

Using several clonal lineages of *Simocephalus vetulus* (Cladocera, Daphniidae) as a random sample, we investigated the genetic component of the halotolerance of one brackish and two freshwater populations of this littoral filter-feeder. We hypothesized that genotypes from the brackish population were more tolerant than freshwater ones, via adaptation to local environmental conditions. Clonal identity was established by a cost-effective molecular fingerprinting technique (MSP-PCR). Two distinct methodologies were used to assess cladoceran sensitivity to synthetic-grade sodium chloride (NaCl): i) standard 48-h acute assays; ii) 12-h survival time (ST) trials. No correlation was found between acute EC_{50} and ST values. The sensitivity of brackish and freshwater clones was comparable in terms of acute EC_{50} (varied from 2.28 g L^{-1} to 3.83 g L^{-1}). On the contrary, genetically-determined differential tolerance to NaCl among populations was found for ST: all brackish genotypes, except one, were more resilient (ST > 120 min) than freshwater clones (ST < 120 min). Bearing in mind that these results were obtained with isolates from the extant population, it is surprising that the range of acute sensitivity of the freshwater and brackish genotypes was similar, and that the only difference between them was the ability of brackish clones to survive longer under high salinity stress (6 g L^{-1}, in ST trials). We must conclude that effect of salinity (original environment context) on the selection of genotypes was weaker than we had expected and than other authors have shown for other stressors.

KEYWORDS

genetic variability; genotype-environment interaction; sodium chloride; halotolerance; *Simocephalus vetulus*

INTRODUCTION

Saline intrusion in freshwaters has received considerable attention (e.g. Schallenberg et al., 2003; Sarma et al., 2006; Gonçalves et al., 2007; Nielsen et al., 2008) justified by the predictions of sea level rise and observation of coastal erosion, which threaten coastal lakes (IPCC, 2008). In coastal lakes that face regular (e.g., tidal) or sporadic salinity increases, freshwater organisms must cope with potentially lethal levels of salinity for short-term periods (hours to days). Particularly for large cladocerans (such as *Daphnia* and *Simocephalus*), their ability to survive (or not) these salinity pulses may modulate zooplanktonic taxa succession (Jeppesen et al., 1994; Schallenberg et al., 2003; Jeppesen et al., 2007; Nielsen et al., 2008) and a lake regime shift (see Jeppesen et al., 2007).

Although sensitive to minor salinity increases (Schallenberg et al., 2003; Sarma et al., 2006; Gonçalves et al., 2007), freshwater cladoceran populations also occur in coastal systems with distinct degrees of connectedness to the sea (e.g. Jeppesen et al., 1994; Schallenberg et al., 2003; Marques et al., 2006; Santangelo et al., 2008). Such physiological plasticity is likely due to genetic factors associated with osmoregulation shifts (Arnér & Koivisto, 1993; Aladin & Potts, 1995; Martínez-Jerónimo & Martínez-Jerónimo, 2007). The degree of phenotypic plasticity to environmental fluctuations is naturally framed within the populations' genetic variability, which is greatly influenced by founder effects and locally-acting selective forces (De Meester, 1996a, 1996b; Boersma et al., 1999; De Meester et al., 2002). Many studies have shown that locally-acting selective forces (particularly predation) lead to the formation of local races or "ecotypes" (local adaptation, see Weider & Hebert, 1987; De Meester, 1996a, 1996b; Boersma et al., 1999; Cousyn et al., 2001; De Meester et al., 2002). This has also been reported in *Daphnia* populations from brackish *versus* freshwater environments (Weider & Hebert, 1987; Teschner, 1995; Barry et al., 2005).

In this study, *Simocephalus vetulus* (Müller, 1776) (Cladocera, Daphniidae) populations inhabiting coastal lakes were used as an experimental model. Our interest was focused on a population inhabiting a brackish lagoon in the South of Portugal, which had to cope with salinities close to 2. Our main goal was to determine whether there is a genetic component of salinity resistance in this population. Since these populations are subjected to salinity pulses, due to the heterogeneous and challenging habitat they live in, they must be able to cope with short-term lethal levels of salinity. Therefore, our aim was to compare the acute halotolerance to salinity (using NaCl as a proxy) of this brackish population with that of freshwater populations of *S. vetulus*. To do so, two distinct methodologies were employed: i) standard acute assays (OECD, 2004), complemented with ii) survival time trials (adapted from Ribeiro et al., 2000; Lopes et al., 2005). We hypothesized that there should be a population differentiation pattern in terms of acute halotolerance, with brackish genotypes being more resilient to NaCl than freshwater ones.

Studies concerning genetic differentiation in quantitative traits of cladocerans have been conducted in daphniids (Cladocera, Daphniidae) living in contaminated vs. uncontaminated habitats (e.g. Lopes et al., 2005; Muyssen et al., 2005) and fish vs. fishless lakes (e.g. De Meester, 1996a; Boersma et al., 1999). Such studies rely on the parthenogenetic mode of reproduction, short life cycle and ease in laboratory culturing of cladocerans (Muyssen et al., 2005), which assure continuous rearing of progeny bearing the genetic information of the founding mothers (De Meester, 1996a; Pfrender & Lynch, 2000). By maintaining clonal lineages under similar laboratory conditions for several generations, environmental and maternal effects can thus be eliminated.

MATERIAL AND METHODS

Cultures and test organisms

Simocephalus vetulus (Müller, 1776) occupies a different ecological niche from most daphniids, due to its habitat preferences and swimming behaviour (Alonso, 1996). It is a macrophyte-associated filter feeder and an important regulator of primary production and nutrient cycling in littoral habitats (Hann, 1995; Alonso, 1996). Clonal lineages of *S. vetulus* were established from three extant populations, which were sampled with oblique trawls of a plankton net. Two of these populations came from coastal freshwater ecosystems in central Portugal, namely a small shallow lake (Lagoa de Mira – LM; lat. 40º26’29’’N, long. 8º45’09’’W) and a nearby semi-artificial ditch or canal (Vala da Fervença – VF; lat. 40º25’36’’N, long. 8º45’19’’W). Although there is hydrological connectivity between these systems and the Ria de Aveiro estuary (i.e. indirect connection with the marine environment via a semi-artificial system of small ditches and channels), salinity does not reach upstream due to a system of small dams and dikes. Thus, there is low dissolved solids concentration and conductivity is typically < 500 μS cm^{-1}. The third population originated from inhabitants of a brackish lagoon (Lagoa de Melides – M; lat. 38º08’01’’N, long. 8º46’52’’W). This coastal lake has an intermittent connection with the sea (usually once or twice a year, either by human or natural action). Samples were taken at the farthest point from the sea entrance, where salinity was 2.3.

Field-collected samples were immediately brought to the laboratory, where a random subsample of ovigerous mothers was isolated from each lake and transferred to 50-mL glass beakers with lake water. As a starting point, we assumed each mother to represent a putatively distinct genotype, until genetic fingerprinting tools clarified clonal identity (see below). Asexually-produced newborns (F_0 generation) from these mothers were used as starting inocula of monoclonal cultures. The genotype collections of *S. vetulus* were reared in moderately hard reconstituted water (pH 7.4-7.8, hardness 80-100 mg $CaCO_3$ L^{-1}, alkalinity 57-64 mg $CaCO_3$ L^{-1}), comprised of: 123 mg L^{-1} $MgSO_4{\cdot}7H_2O$, 96 mg L^{-1} $NaHCO_3$, 60 mg L^{-1} $CaSO_4{\cdot}2H_2O$, and 4 mg L^{-1} KCl, *sensu* ASTM (1980) and USEPA (2002). This synthetic medium was supplemented with 4 mL L^{-1} of a standard organic additive (algal extract as described by Baird et al., 1989) and vitamins (as described for M4 and M7 medium - Elendt & Bias, 1990). Cyclically, neonates from the 3rd or 4th brood were isolated and their mothers discarded, thus providing continuous culture renewal (F_1, F_2, F_3, …) for at least 10 generations. This provided a required refractory period, ensuring normalisation of environmental and maternal influences; both freshwater and brackish genotypes were cultured in the described freshwater reconstituted medium. Cultures were reared under a temperature of 20±2ºC and a $16h^L{:}8h^D$ photoperiod, and organisms were fed three times a week (Monday, Wednesday, Friday) with a *Pseudokirchneriella subcapitata* ration of 1.5×10^5 cells mL^{-1} (for more information on algal culture and ration, see Antunes et al., 2004). Vessels with acclimated animals were used as stock cultures for all subsequent experiments.

Clonal identification

MSP-PCR (microsatellite-primed polymerase chain reaction) or ISSR (inter-simple sequence repeats) is a fast and simple genotyping technique that uses repetitive and highly variable regions of the genome (microsatellites) to design universal primers, which produce highly variable fingerprinting profiles (Zietkiewicz et al., 1994; Karp & Edwards, 1997; Abbot, 2001). This technique has shown to be useful in studies on systematics and in the differentiation of strains and breeds within the same species (e.g. Alves et al., 2007) and within cell lineages from the same individual (e.g. Grasela & McIntosh, 2003).

DNA was extracted individually from preserved specimens (in 70-80% ethanol) of each population using a standard protocol (Schwenk et al., 1998), which has undergone minor adjustments over time (see Brede et al., 2006). To maximise extraction, only adult females were used and a preliminary step consisting of specimen maceration was included in the extraction procedure. All monoclonal cultures successfully established in the laboratory were analysed, in a total of 54 putative lineages (18 per population).

Each MSP-PCR reaction was performed in a total volume of 24 µL, containing 2.0 mM $MgCl_2$, 0.2 mM dNTP, 1x PCR Buffer, 0.3 µM primer, and 1U of DNA Polymerase (Go Taq Flexi DNA polymerase, PROMEGA). Three primers [$(GTG)_5$, $(CAG)_6$, $(ACC)_6$] were chosen to discriminate genotypes, after preliminary testing on PCR conditions and reproducibility of a wider battery of primers (see Abbot, 2001; Grasela & McIntosh, 2003; Dusinský et al., 2006; Alves et al., 2007). Cycling conditions were as follows: i) a denaturation step consisting of one cycle of 120 s at 95ºC; ii) 35 cycles of 45 s at 94ºC, 45 s at 50º or 57ºC (depending on the primer used), and 90 s at 72ºC; iii) a final elongation step of 10 min at 72ºC. Primer $(GTG)_5$ required an annealing temperature of 50ºC, while $(CAG)_6$ and $(ACC)_6$ were amplified at 57ºC. PCR products were separated on 1.5% agarose gels (run at 90 V for 4 h) and visualized under UV light, after immersion of gels in an ethidium bromide solution for 10 min. A PROMEGA loading buffer (Go Taq Flexi) was used and a 100 bp molecular marker (100-1500 bps) was loaded in the gel as a reference.

The analysis of genetic variability among individuals was performed by comparison of the banding patterns obtained. The patterns were converted to a bidimensional binary matrix (1 = presence of band, 0 = no band). Samples were always run twice (amplification and fragment separation) and bands were only scored “1” if present in both amplifications. From this initial matrix, we derived similarity matrices using the Jaccard’s coefficient (Culley, 2005), which allowed us to build dendrograms reflecting the genetic architecture of populations sampled. The dendrograms were constructed using UPGMA (Unweighted Pair Group Method with Arithmetic mean). All analyses (gel and fingerprint data) were performed with GelCompar II (Applied Maths, USA), using its default optimisation and tolerance levels. We used a conservative cut-off value (> 30% dissimilarity) to differentiate clonal lineages, based on previous optimisation of PCR consistency.

Halotolerance assays

Standard acute assays were conducted by exposing distinct *S. vetulus* clonal lineages (M63, M48, M66, M13, M7, M50, M20, M36, LM2, LM38, LM5, LM48, LM42, LM41, LM64, LM25, VF7, VF35, VF18, VF70, VF29, VF48, VF69) to sodium chloride (NaCl) aqueous solutions, following standard protocols (ISO, 1996; USEPA, 2002; OECD, 2004). A stock solution (50 g L^{-1}) was prepared by dissolving analytical grade NaCl in moderately hard reconstituted water. Test solutions (Table 1) were then obtained by diluting the appropriate volumes of stock solutions in the synthetic medium. Salinity and conductivity measurements were performed with a WTW conductivity meter. A fixed volume (10 mL of test solution) was added to the corresponding test vessels. Neonates (less than 24 h old and born between the 3rd and the 5th brood) were exposed to a geometric sequence of NaCl concentrations (2.0-5.0 g L^{-1}; Table 1), under the same conditions of rearing cultures. The range of concentrations used was selected based on preliminary tests and toxic levels reported in the literature (e.g. Gonçalves et al., 2007). Our goal was to find a range of concentrations which could be applied to all clones. Four replicates per treatment were used and five neonates were randomly assigned to each 10-mL replicate (i.e. a total of 20 organisms per test concentration were used). In all tests, a negative control was used, where no NaCl was added (nominal concentration 0.0 g L^{-1}; Table 1). Vessels were screened for immobilised daphniids after a 48 h exposure period. During these acute exposures, neither organic extract nor food was added.

Survival time trials were conducted with the same clonal lineages as the standard acute assays; experimental procedures were adapted from Ribeiro et al. (2000) and Lopes et al. (2005). Neonates (less than 24 h old and born between the 3rd and the 5th brood) were exposed to a fixed concentration of NaCl (6.0 g L^{-1}), which is normally lethal within 12 h. Salinity and conductivity measurements were also performed (Table 1). Assays were performed in 24-well cell culture plates, where six organisms of each clone were individually exposed to 2 mL of test medium (moderately hard reconstituted water spiked with NaCl). Four genotypes could thus be tested in each plate. For each clone, trials were performed in triplicate, with each replicate consisting of neonates produced in different days or from different culture flasks of the same clone. Organisms were observed every 15 min from the beginning of the trial until 180 min, every 30 min from 180 min to 360 min, every 1 h from 360 min to 720 min, and, if necessary, every 2 h after 720 min (12 h). Similarly to the standard acute assays (see above), we used immobilisation as the trial endpoint. Organisms were scored as immobile under the stereoscope (8-10x magnification) if they were not able to swim after gentle and repeated prodding. Trials ended when all organisms were considered as immobilised in two consecutive observations. In parallel to all survival time trials, controls (neonates exposed to unspiked culture medium) were performed, following the same experimental design; trials were valid only if no immobilised individuals were recorded in the control at the end of the test.

Table 1 – Nominal concentrations (*) of sodium chloride used in the halotolerance assays and respective salinity and electrical conductivity values in respective test media (mean of three measurements ± standard deviation).

Assays	[NaCl]* g L^{-1}	Salinity	Conductivity (μS cm^{-1})
Acute assays	0.0	0.0 ± 0.00	216.4 ± 44.75
	2.0	2.1 ± 0.06	3650 ± 100.0
	2.4	2.5 ± 0.00	4323 ± 40.4
	2.9	3.0 ± 0.06	5127 ± 41.6
	3.5	3.7 ± 0.06	6047 ± 35.1
	4.2	4.4 ± 0.10	7157 ± 127.4
	5.0	5.2 ± 0.17	8333 ± 203.1
Survival-time trials	6.0	6.3 ± 0.06	9977 ± 98.2

* The contribution of the test medium in terms of Na^+ and Cl^- was assumed to be unimportant, given the small amount of these ions in its composition (see text).

Statistical analysis

Acute toxicity was expressed as 48 h immobilisation EC_{50} values (and respective 95% confidence intervals), as estimated with Probit analysis using Minitab® software. Average survival time (ST) for each replicate and corresponding acute EC_{50} values were integrated in the computation of a halotolerance index (HI):

$$HI = 100 \text{ x } relST \text{ x } relEC_{50},$$

where relST and $relEC_{50}$ represent the relative ST or EC_{50}, i.e. the value of the clone under consideration divided by the maximum of all clones. This normalisation step was introduced in the calculation to give similar weight to both variables, while allowing HI to vary between 100 (extreme tolerance) and close to 0 (extreme sensitivity). This weighed metric is thus a simple way to order genotypes in terms of their sensitivity.

ST was analysed for differences between populations, following a nested ANOVA design (mixed-effects model), with population as the fixed factor and clone (nested within population) and replicate trial (nested within clone) as the random factors. Individual survival time measurements constituted the error term of the ANOVA. No replicates per clone were available for EC_{50} values, so a one-way ANOVA was used to assess differences among *Simocephalus* populations. All analyses were computed using Minitab® software, which also allowed extracting variance components for each source of variation.

RESULTS

MSP-PCR band profiles allowed the differentiation of the three populations (Figure 1). The only exception was a subset of six LM genotypes, which was associated with the M population cluster. For this study, 23 clonal lineages (8 clones for M, 8 clones for LM and 7 clones for VF) were used in the experiments (see list in Table 2), after scored as genetically distinct genotypes (Figure 1; the most similar genotypes used were M20 and M36). Genetic identity was confirmed by additional dendrograms for each population (data not shown), using a pre-determined cutoff level of 70% similarity. This allowed a security margin in the discrimination of clones.

The acute immobilisation EC_{50} values (Table 2 and Figure 2) ranged from 2.28 g L^{-1} to 3.83 g L^{-1}, thus demonstrating a large range of halotolerance in the tested genotypes. Comparing clone sensitivity within each population, the EC_{50} values obtained ranged from 2.48 g L^{-1} to 3.83 g L^{-1} in population M (brackish lagoon), 2.81 g L^{-1} to 3.59 g L^{-1} for population LM (freshwater lake), and 2.28 g L^{-1} to 3.60 g L^{-1} in population VF (freshwater ditch). These ranges show an overlap of the values of halotolerance between populations (see distribution of clones in the x axis of Figure 2), confirming no differentiation between populations (one-way ANOVA: d.f. = 2, 20 ; F = 0.48; $p = 0.63$). Comparing all clones, the lowest (2.28 g L^{-1}) EC_{50} was observed in VF (a freshwater population), while the highest values (3.83 and 3.65 g L^{-1}) were recorded in clones from the brackish population (Table 2 and Figure 2).

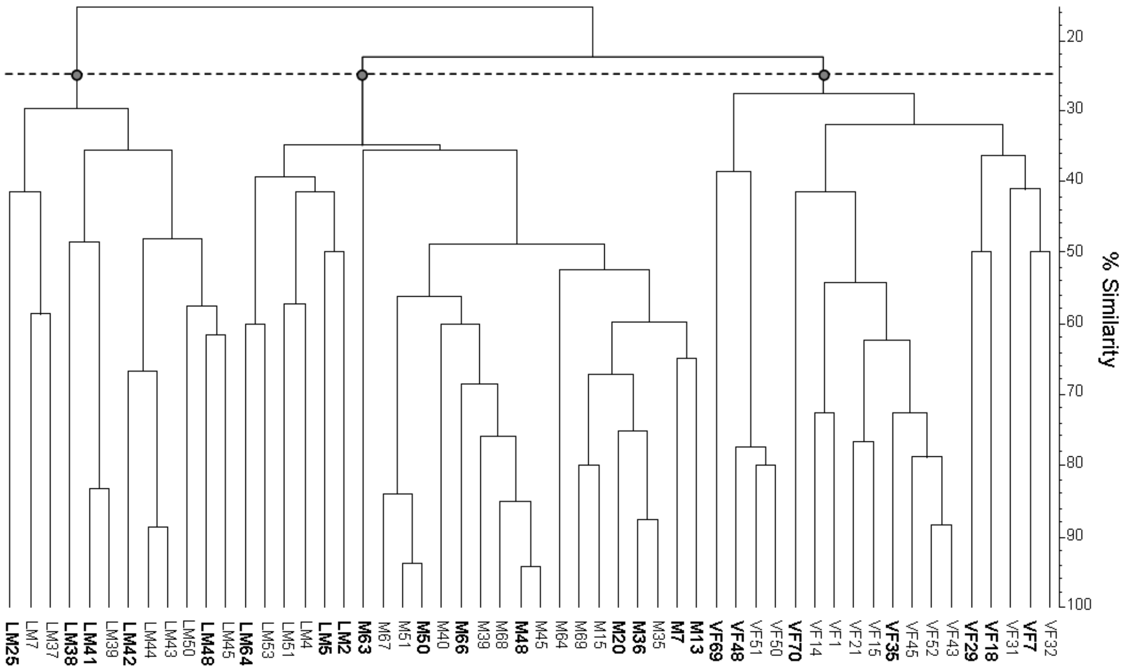

Figure 1 – Dendrogram of fingerprinting patterns of different individuals of *Simocephalus vetulus* from Melides lagoon (M), Mira lake (LM) and Fervença ditch (VF). Clones used in the experiments are shown in boldface.

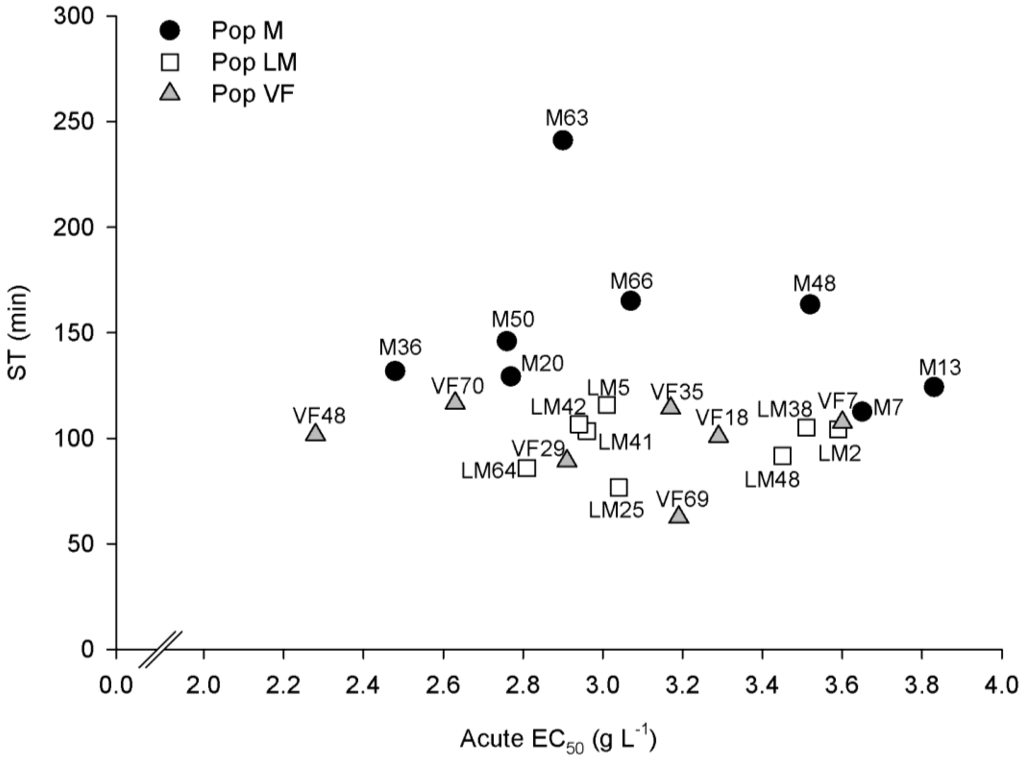

Figure 2 – Relative halotolerance of *Simocephalus vetulus* genotypes from three populations (M, LM, and VF): acute EC$_{50}$ values *versus* survival time (ST). Each circle, square, and triangle represents a genotype.

Table 2 – Acute immobilisation EC_{50} values (and respective 95% confidence intervals), survival time (ST ± standard error), and halotolerance index (HI ± standard error) for *Simocephalus vetulus* populations exposed to sodium chloride. For each population, clones are sorted by descending HI (i.e. most tolerant clones on top).

Population	Clone	Acute EC_{50} (g L^{-1})	ST (min)	HI
M (brackish)	M63	2.90 (2.61-3.17)	241.0 ± 41.77	64.0 ± 6.91
	M48	3.52 (3.31-3.77)	163. 3 ± 18.70	52.7 ± 4.66
	M66	3.07 (2.76-3.38)	165.0 ± 17.45	46.4 ± 3.87
	M13	3.83 (3.63-4.05)	124.2 ± 19.13	43.6 ± 8.36
	M7	3.65 (3.43-3.90)	112.5 ± 8.68	37.6 ± 2.55
	M50	2.76 (2.55-2.96)	145.8 ± 13.33	36.9 ± 4.28
	M20	2.77 (2.59-2.95)	129.2 ± 4.40	32.8 ± 0.76
	M36	2.48 (2.22-2.70)	131.7 ± 10.88	29.9 ± 2.63
LM (freshwater)	LM2	3.59 (3.38-3.84)	104.2 ± 10.74	34.3 ± 6.01
	LM38	3.51 (3.31-3.75)	105.0 ± 7.70	33.8 ± 4.13
	LM5	3.01 (2.76-3.26)	115.8 ± 10.56	31.9 ± 5.34
	LM48	3.45 (3.20-3.73)	91.7 ± 7.85	29.0 ± 1.15
	LM42	2.94 (2.82-3.10)	106.7 ± 10.21	28.7 ± 3.02
	LM41	2.82 (2.52-3.04)	103.3 ± 8.12	28.0 ± 4.07
	LM64	2.81 (2.65-2.99)	85.8 ± 8.98	22.1 ± 3.51
	LM25	3.04 (2.83-3.28)	76.7 ± 5.93	21.4 ± 1.86
VF (freshwater)	VF7	3.60 (3.39-3.84)	107.5 ± 8.34	35.5 ± 5.30
	VF35	2.74 (2.09-2.81)	114.2 ± 12.49	33.2 ± 4.91
	VF18	3.29 (3.08-3.52)	100.8 ± 8.03	30.4 ± 4.23
	VF70	2.63 (2.35-2.87)	116.7 ± 12.22	28.1 ± 3.69
	VF29	2.91 (2.72-3.15)	89.2 ± 10.32	23.8 ± 1.56
	VF48	2.28 (1.50-2.55)	101.7 ± 18.53	21.2 ± 2.70
	VF69	3.19 (3.02-3.38)	62.5 ± 5.60	18.3 ± 2.77

The EC_{50} values were not consistent with the halotolerance of clones in survival time (ST) trials. In fact, the correlation between these variables (Figure 2) was weak and not significant ($r = -0.083$; $p = 0.71$). A good example of this lack of correspondence is clone M63 (Figure 2 and Table 2), which obtained an extreme ST value (241 min) but whose EC_{50} was in the middle region of the plot (2.9 g L^{-1}). Comparing clone sensitivity within each population, the ST values obtained ranged from 112.5 min to 241 min in population M (brackish lagoon), 76.7 min to 115.8 min for population LM (freshwater lake), and 62.5 min to 116.7 min in population VF (freshwater ditch). These intervals showed that brackish clones were more halotolerant than the freshwater clones. The distribution of clones along the y axis of Figure 2 shows that ST of freshwater genotypes was always below 120 min, while all brackish clones – except M7 (ST = 112.5 min) – displayed STs above this threshold. In average, clones from the brackish population (M) resisted 1 h more than the two freshwater populations (Figure 3). This produced statistically significant differences among populations (Table 3), despite intra-populational (clonal) variability. Indeed, inter-populational variability was higher than the within population variance component (17% *versus* 10%; Table 3).

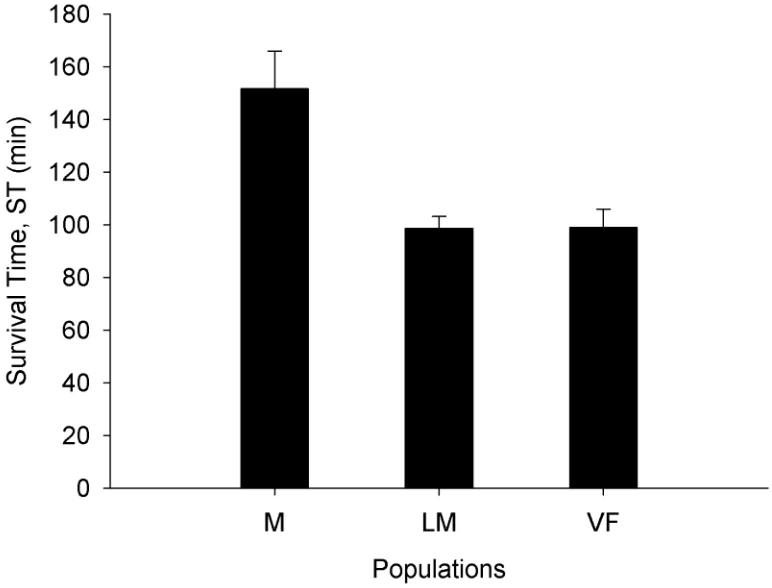

Figure 3 – Mean survival time (ST) for each of the three populations studied (M, LM, and VF). Error bars represent standard error.

Halotolerance index (HI) values ranged from 29.9 to 64.0 for population M (brackish lagoon), 21.4 to 34.3 for population LM (freshwater lake), and 18.27 to 35.45 in population VF (freshwater ditch). Consistently with ST values, 6 out of 8 genotypes from the brackish population scored the highest HI values (M20 and M36 were the exception) of all tested genotypes.

Table 3 – Nested ANOVA summary tables for survival time (ST), including variance components (% Var). ANOVA model includes a fixed factor (Pop – population) and two (Clone and Rep – replicate) nested random factors.

	Model	df	Adj MS	F	p	% Var
ST	Pop	2	130938	9.88	0.001	16.8
	Clone (Pop)	20	13254	3.48	<0.001	10.4
	Rep (Clone)	46	3807	1.04	0.412	0.45
	Error	345	3670			

DISCUSSION

Our study reports the differential tolerance to NaCl between one brackish and two freshwater populations of *S. vetulus*. All except one brackish genotypes were able to cope with high salinity stress (6 g L^{-1}) for a longer period than freshwater genotypes, in survival time (ST) trials. Since most maternal and environmental influences were previously discarded (by culturing clones for several generations under standardized conditions), these differences are assumed to be genetically determined. Therefore, this population differentiation pattern is shaped by locally-acting selective forces, in this case salinity. The increased resilience of brackish clones to high salinity levels, expressed as increased survivorship, is in agreement to what was found by Weider and Hebert (1987) and Teschner (1995) for *Daphnia*. Local selective forces play an important role in the genetic structure and diversity of invertebrate populations, especially those with reduced dispersal capabilities (De Meester, 1996b; De Meester et al., 2002; 2006). Thus, even minor salinity increments in freshwaters (of a progressive, long term nature) may cause subtle impacts in animal populations, such as the appearance of locally-selected races or “ecotypes” (sensu Weider & Hebert, 1987).

Unlike ST trials, no population differentiation pattern was found with standard acute assays. These contradictory results are portrayed by the lack of correspondence between acute (48 h) EC_{50} and survival time . In a work with *D. longispina*, Lopes et al. (2005) observed positive correlations between LT_{50} (lethal time) and LC_{50} (lethal concentration) only with copper, but not for zinc, cadmium, or hydrogen ions. Their original population was historically exposed to acid mine drainage contaminants, including copper and low pH. The lack of association between moderately lethal and extremely lethal levels of stressors, such as metals or pH(in the study by Lopes et al. (2005) and NaCl (in our study), has an unknown biological meaning. High stressor levels trigger fast-response genes, such as hormones or protective enzymes, which modulate behavioural and physiological responses to increase resistance to environmental stress (see Hoffmann & Parsons, 1993). In the case of metals and hydrogen ions – and probably NaCl – animals die out of a series of related complications besides ionoregulatory failure (Wilson & Taylor, 1993). Hence, the regulation mechanisms to moderately or extremely lethal levels of toxicants should be the same; thus, other explanations must be at the basis of the contradictory results observed for ST trials and standard acute assays. One hypothesis is that the lack of association between both endpoints is related to their temporal resolution (see below).

Differences in ST between freshwater and brackish populations were observed in a limited time scale, varying from minutes to a few hours (between 35 min to 178 min). Differences in sensitivity at such a time scale are not perceptible in standard acute exposures. In ST trials, a lethal concentration of NaCl is used and frequent measurements are taken, while in standard acute assays the organisms are checked at larger time intervals (24-48 h) after exposure to a range of increasing concentrations. There is considerable loss of information when daphniids are screened at such large time intervals (≥ 24 h). Thus, the finer temporal resolution of ST trials seems theoretically more advantageous and sensitive than standard acute exposures for exploring differentiation in quantitative genetic traits. Also, ST allows one to analyse the data using an ANOVA approach and to partition associated sources of variation (as done here), which is powerful and useful.

In this study, field clones were isolated from extant populations. Alternatively, many authors (e.g. Teschner, 1995; De Meester, 1996a; Boersma et al., 1999; Barry et al., 2005) use resting eggs (which result from sexual reproduction) as source of isolates. Both the active and dormant population have been subjected to different selective forces in each habitat throughout generations (De Meester, 1996b; De Meester et al., 2002), with emphasis on salinity in this case. The active population is less diverse, as it is constituted by individuals that have been subjected to local forces also during their ontogeny (De

Meester et al., 2006), passing through various bottlenecks (hatching success, ability to feed and grow, asexual reproductive success). Therefore, the genotypes used in this study are the most adapted to the specific habitat of origin (freshwater or brackish). Thus, it is surprising that the range of acute sensitivity of the isolated freshwater and brackish genotypes is similar, with the only difference between them being the ability of brackish clones to survive longer under high salinity stress (6 g L^{-1}). This is contrary to the findings of Lopes et al. (2004, 2005), who found remarkable differences between the acute sensitivities to copper (using standard acute assays) of historically-stressed and reference populations. We must conclude that the effect of salinity (original environment context) on the selection of genotypes was weaker than we had expected and than other authors have shown for other stressors.

When genotypes are isolated from the extant population, such as the case, the use of molecular fingerprinting is essential to assure clonal identity, since active populations are a mix of parthenogenetic and sexual clones (De Meester et al., 2006). In this study, we used a simple and cost-effective technique (MSP-PCR), which allowed satisfactory discrimination of genotypes (clones), analogously to RAPD (e.g. Weber & Declerck, 1997), microsatellite (e.g. Chopelet et al., 2008), or allozyme (e.g. Weider & Hebert, 1987) analyses. However, a conservative approach was needed, both in terms of band profile analysis and dissimilarity cut-off level, in order to avoid false positives (i.e. scoring identical genotypes as distinct). A reproducibility analysis of the technique showed that caution is needed because of variability among amplifications (probably due to misprints during PCR). Nonetheless, a few markers allowed the successful differentiation of the three studied populations (except for a small group of LM genotypes), even among populations with some connectivity degree (LM and VF). Although this was not the purpose of the study, we foresee a potential application of this technique in the decomposition of the genetic architecture of daphniid natural populations, provided a more comprehensive set of markers is used and that the technique's reproducibility and amplification specificity are improved.

Acute EC_{50} for *S. vetulus* genotypes ranged between 2.3 g L^{-1} to 3.8 g L^{-1} of NaCl in this study. This is in agreement with the microcosm experiments of Sarma et al. (2006), whose *S. vetulus* experimental populations barely survived at 4 g L^{-1}, but were able to reproduce up to a concentration of 2 g L^{-1}. Halotolerance values for other cladocerans have also been reported using laboratory assays. Martínez-Jerónimo and Martínez-Jerónimo (2007) reported a 48 h LC_{50} of 5.48 g L^{-1} for *D. magna*, while demonstrating that acclimated organisms could survive and reproduce in NaCl concentrations up to 6 g L^{-1}. Gonçalves et al. (2007) reported an acute 48 h EC_{50} of 2.9 g L^{-1} and 5.9 g L^{-1} for *D. longispina* and *D. magna*, respectively. These data suggest that *S. vetulus* halotolerance, regardless of their habitat of origin (brackish versus freshwater), is comparable to that of *D. longispina*, and both are more sensitive than *D. magna*. Indeed, the latter species is documented to be halotolerant and is a common inhabitant of brackish lakes and rock pools (Arnér & Koivisto, 1993; Teschner, 1995; Alonso, 1996). *Ceriodaphnia lacustris*, *D. thomsoni*, *D. pulex* and *D. pulicaria* have also been found in habitats with moderate (5-13) salinities (Weider & Hebert, 1987; Barry et al., 2005; Northcote & Hall, 2010). However, most cladocerans are restricted to freshwater (Alonso, 1996) and are only able to cope with low salinities (usually < 2; see Sarma et al., 2006) (for field data, see: Jeppesen et al., 1994; Schallenberg et al., 2003; Marques et al., 2006; Santangelo et al., 2008). In fact, Schallenberg et al. (2003) predicts that severe alterations of zooplankton community structure and abundance may occur at salinities > 1.2. These authors found *Simocephalus* sp. to tolerate salinities up to 1.2, while in our study *S. vetulus* occurred in Melides (population M) at a salinity of 2.3. However, we only found partial evidence that this population possesses genetically-determined tolerance mechanisms to endure saline stress, as portrayed by the lack of correspondence between standard acute assays (tolerance overlap) and survival time (population differentiation).

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Capítulo II

Life-history responses of salinity-tolerant and salinity-sensitive lineages of a stenohaline cladoceran do not confirm clonal differentiation

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O presente capítulo constitui um manuscrito em publicação em revista internacional com arbitragem científica "Hydrobiologia" e é, por esse motivo, apresentado na língua inglesa.

Life-history responses of salinity-tolerant and salinity-sensitive lineages of a stenohaline cladoceran do not confirm clonal differentiation

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ABSTRACT

The adaptability of freshwater organisms to brackish conditions is important to understand the resilience of freshwaters to saline intrusion, a phenomenon that may affect vulnerable freshwater habitats. Bearing this in mind, this work intended to assess if there are genetically-determined differences in the tolerance of stenohaline cladocerans in 21 d exposure scenarios to sodium chloride (NaCl, as a proxy for salinity). The objective was to compare the reproductive output of salinity-tolerant (brackish) versus salinity-sensitive (freshwater) *Simocephalus vetulus* genotypes, obtaining reaction norms for quantitative phenotypic traits (survival, fecundity, fitness). We found intra-specific (i.e. clonal) variability in the life history responses of *S. vetulus* populations, but no evidence that the most resistant clones (brackish) were best suited to cope with salinity. Surprisingly, brackish genotypes were the most affected in terms of fecundity when exposed to sublethal levels of NaCl, which could be viewed as a potential trade-off mechanism. Although differences in tolerant and less tolerant genotypes were small, they may be important in genotype and species sorting in colonization or recolonization events in freshwater systems facing salinization.

KEYWORDS

Simocephalus vetulus; halotolerance; genetic variability; local environmental conditions; life history traits; sodium chloride

INTRODUCTION

Cladocerans have been documented as being mostly restricted to freshwater environments (Arnér & Koivisto, 1993; Alonso, 1996; Bailey et al., 2004; Sarma et al., 2006), although they can be found and reproduce in brackish waters (Teschner, 1995; Santangelo et al., 2008; Northcote & Hall, 2010). This is possibly due to their osmorregulatory capacity, which allows them to resist to minor changes of salinity (Arnér & Koivisto, 1993; Martínez-Jerónimo & Martínez-Jerónimo, 2007). Indeed, the phenotypic plasticity of these crustaceans allows them to adapt their life history and physiology when facing new environmental conditions (Weber & Declerck, 1997; Castro et al., 2007). On the long term, cladoceran populations also have the ability to adapt to environmental changes, via microevolutionary processes (Cousyn et al., 2001; Declerck & Weber, 2003; Van Doorslaer et al., 2007).

Under brackish conditions, genotypes from brackish environments are expected to be more halotolerant, thus surviving and reproducing better under saline stress than genotypes from freshwater habitats (Teschner, 1995; Barry et al., 2005). However, confirmation of local adaptation in brackish populations has so far been shown in cladoceran species that can tolerate intermediate levels of salinity (up to 13), such as *D. pulex* (Weider & Hebert, 1987), *D. thomsoni* (Barry et al., 2005) and *D. magna* (Teschner, 1995). No such evidence exists for less resistant taxa, such as *Hyalodaphnia* (e.g., *D. galeata*, *D. longispina*) or *Simocephalus*, which are common freshwater pond and lake dwellers. In part, the reason for this is that these taxa (poor osmorregulators) tend to disappear at the onset of salinization, as they are only able to cope with low salinities (usually < 2; see Sarma et al., 2006) (for field data, see: Jeppesen et al., 1994; Schallenberg et al., 2003; Marques et al., 2006; Santangelo et al., 2008).

In a previous study (Loureiro et al., 2012) using genotypes from a brackish population and two freshwater ones of *Simocephalus vetulus* (Cladocera, Daphniidae), we demonstrated that their acute halotolerance was genetically determined. After removing environmental and maternal effects, we found that all - except one - brackish genotypes were more tolerant to lethal levels of sodium chloride (NaCl, 6 g L^{-1}) than freshwater genotypes, in terms of survival time (measured as time to immobilization). However, no correspondence was found between this endpoint and the standard acute EC_{50}. We concluded that, although present, local adaptation to the brackish environment was not as strong as shown in other studies with daphniids.

Bearing this in mind, this work focused on a subset of clones from Loureiro et al. (2012) to assess if the determinants of halotolerance are observable in long term (chronic) exposure scenarios. Our objective was to evaluate the reproductive output of salinity-tolerant (brackish) *versus* salinity-sensitive (freshwater) *S. vetulus* genotypes, obtaining reaction norms for quantitative phenotypic traits (survival, reproduction, and *per capita* intrinsic rate of increase, *r*). We intend to respond to the following questions: 1) do salinity-tolerant (brackish) genotypes perform better than freshwater clones under low saline concentrations?; 2) if so, do brackish genotypes lose their original freshwater adaptation (*sensu* Teschner, 1995), i.e. do they perform better under saline conditions than in freshwater?; 3) is there potential to adapt to saline intrusion in freshwater populations?

MATERIAL AND METHODS

Cultures and test organisms

Clonal lineages of *Simocephalus vetulus* (Müller, 1776) (Cladocera, Daphniidae) were established from three extant populations. All populations came from freshwater ecosystems (Lagoa de Mira – LM; Vala da Fervença – VF; Lagoa de Melides – M), with one of them (M) receiving intermittent salinity intrusions (see Loureiro et al., 2012). The *S. vetulus* genotype collections were cyclically reared in moderately hard reconstituted water (123 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 96 mg L^{-1} of $NaHCO_3$, 60 mg L^{-1} $CaSO_4 \cdot 2H_2O$, e 4 mg L^{-1} KCl, *sensu* ASTM (1980) and USEPA (2002)), supplemented with 4 mL L^{-1} of a standard organic additive (algal extract) and vitamins (for further details, see Loureiro et al., 2011; Loureiro et al., 2012). Cultures were maintained under a temperature of 20±2ºC and a $16h^L$:$8h^D$ photoperiod, and organisms were fed three times a week (Monday, Wednesday, Friday) with a *Pseudokirchneriella subcapitata* ration of 1.5×10^5 cells mL^{-1} (for more information on algal culture and ration, see Antunes et al., 2003; Antunes et al., 2004).

In this work, we used a subset of the genotypes described by Loureiro et al. (2012), by selecting five clones among the most sensitive to NaCl (all from the freshwater populations, LM and VF) and five others among the most resistant to NaCl (all from the brackish population, M). The halotolerance index (HI, a weighted metric of survival time and standard acute EC50; Loureiro et al., 2012) was used as a measure of sensitivity (it varies from 0 to 100). The following clones were used (HI is shown in brackets): LM25 (21.4), LM41 (28.0), LM64 (22.1), VF29 (23.8), VF69 (18.3), M7 (37.6), M13 (43.6), M48 (52.7), M63 (64.0), and M66 (46.4).

Life history experiments

Neonates of each clone (less than 24 h old and born between the 3rd and the 5th brood) were exposed to a sequence of NaCl concentrations (0, 0.25, 0.50, 1.0, 1.5 and 2.0 g L^{-1}), for 21 d, to evaluate their life history responses to this stressor. The dominant ions in seawater are chloride (Cl^-) and sodium (Na^+), which makes NaCl a suitable and convenient proxy for salinity (see Sarma et al., 2006; Martínez-Jerónimo & Martínez-Jerónimo, 2007), instead of seawater or commercial sea salt. Nominal NaCl concentrations are expressed throughout the manuscript in g/L, while practical salinity readings in the field are unitless (UNESCO, 1985). The solutions were obtained by dissolving NaCl in culture medium (see above and Loureiro et al., 2012). Ten replicate vessels per concentration were used, with each vessel (with 50 mL of test solution) containing one individual. All experiments were carried out under the same conditions described for cultures, except for the following: test solutions were renewed twice a week and organisms were fed daily 0.75×10^5 cells mL^{-1} of *P. subcapitata*. Survivorship and reproductive output (cumulative fecundity, and number of broods per female) were calculated from daily records. Additionally, we measured body length at day 7 and calculated individual somatic growth rates, according to the following expression:

$$\text{growth rate} = \frac{\ln(l_\mathrm{f}) - \ln(l_\mathrm{i})}{\Delta \mathrm{t}},$$

where Δt is the time interval (7 d), l_f is the body size of the daphnids at day 7, and l_i is the initial body length – measured in a subsample (n=20) of neonates. Somatic growth rate is expressed in d^{-1}.

Survival and fecundity estimates were used to compute the *per capita* rate of population increase (r, d^{-1}), using the Euler-Lotka equation:

$$1 = \sum_{\mathrm{x}=0}^{n} \mathrm{e}^{-r\mathrm{x}}\, l_\mathrm{x} m_\mathrm{x}\ ,$$

where x stands for age class (d), l_x is the probability of surviving to age x, and m_x represents age-specific fecundity. This demographic parameter was calculated after pooling the life-history data of all replicate vessels in each tested concentration; thus, for statistical purposes, replicate pseudo-values for *r* were generated with the jack-knifing technique described by Meyer et al. (1986).

Statistical analysis

Several measures of chronic halotolerance were derived from life history data, for each clone. The reproductive EC_{50} was obtained using probit analysis, after calculating % reduction in mean fecundity relatively to the control treatment (0 g L^{-1}). The NOEC (no observed effect concentration) for fecundity, somatic growth rate, and intrinsic rate of increase (*r*) was obtained by assessing differences between NaCl concentrations and the control treatment, using an ANOVA approach (one-way ANOVA followed by Dunnett's test). The decrease in fitness was expressed as the slope of the regression line of *r* as a function of NaCl concentration. Because most clones did not survive in the highest concentration (2 g L^{-1}), we did not consider it in the estimation of the slope. Analogously, this regression approach was used to express the decrease in fecundity, growth, and survival. In the latter case, survival data were a priori arcsine-transformed to correct for non-normality: $x' = \arcsin(\sqrt{x})$.

Correlation and regression analyses were used to assess relationships among halotolerance measures from life history data, as well as between these and acute halotolerance measures (survival time and standard acute EC50, from Loureiro et al., 2012). One-way ANOVAs were also employed to halotolerance measures from life history data in order to test for differences between salinity-sensitive and salinity-tolerant genotypes.

Principal components analysis (PCA) was used to reduce the multivariate matrix to a bidimensional plot, thus allowing to position genotypes in terms of their halotolerance as a function of the full set of chronic and acute (EC_{50} and ST) halotolerance measures. Prior to PCA, halotolerance data were standardized to reduce scale effects. PCA scores (dimensions 1 and 2) for each genotypes were analyzed with one-way ANOVAs to assess differences between salinity-sensitive and salinity-tolerant genotypes.

RESULTS

The selected genotypes were usually affected from 0.5-1 g L^{-1} onwards, regarding their survival, growth, reproduction and rate of population increase. For simplification purposes, we only show data on fecundity and survival (Figure 1) and rate of population increase (r, Figure 2). Most clones survived up to 1.5 g L^{-1}; noticeable exceptions were clones LM25 (survived only until 1.0 g L^{-1}) and VF69, M7 and M13 (survived up to 2 g L^{-1}) (Figure 1). Fecundity and growth decreased monotonically with increasing NaCl concentration, and their impairment occurred at concentrations at which mortality was already recorded (Figure 1).

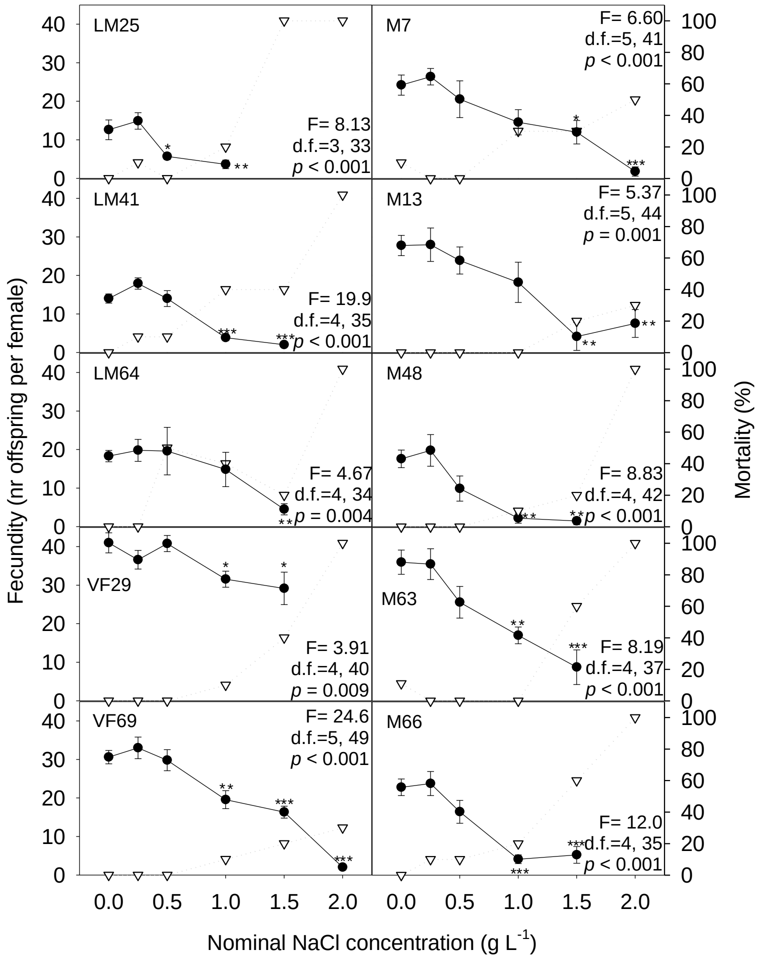

Figure 1 – Mean fecundity (offspring per female; black line) and mortality (%; grey line) of *S. vetulus* genotypes exposed to increasing concentrations of sodium chloride (NaCl). Error bars represent standard error, and asterisks assign differences between nominal NaCl concentrations and negative control for fecundity (one-way ANOVA followed by Dunnet test; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). Left and right panels represent freshwater and brackish genotypes, respectively.

The shape of the toxicity curves (fecundity, somatic growth rate and mortality vs. nominal NaCl concentrations) revealed diverse halotolerance profiles among genotypes (Figures 1 and 2). This was reflected in the chronic halotolerance measures for each genotype (summarized in Table 1) and in the comparative analysis of their population growth rate (measured as *r*; Figure 2). NOEC values for *r* (Table 1), obtained with an ANOVA approach, revealed that clone LM25 was the most sensitive, while clones M7 and M13 were the most resilient to NaCl. The decrease in fitness (slope of the relationship between fitness and NaCl concentration) was strongly related to the reproductive EC$_{50}$ (Figure 3). Again, a pattern of genotype differentiation is visible (Figure 3): clones VF29, VF69 and M7 were the most halotolerant, followed closely by LM64 and M13; clones M48, LM25, M66, LM41, and LM63 were the most sensitive to NaCl.

Table 1 – Chronic halotolerance measures for each *Simocephalus vetulus* genotype after 21 d exposure to increasing NaCl concentrations. Confidence limits (95%) for reproductive EC$_{50}$ are shown in brackets.

Origin	Clone	Reproductive EC$_{50}$ (g L^{-1})	Survival at 1.5 g L^{-1}	NOEC fecundity (g L^{-1})	NOEC growth (g L^{-1})	NOEC r (g L^{-1})
Freshwater	LM25	0.629 (0.562-0.695)	0.00	0.25	0.25	0.25
	LM41	0.979 (0.917-1.04)	0.60	0.50	0.50	0.50
	LM64	1.28 (1.216-1.333)	0.80	1.0	0.50	1.0
	VF29	1.44 (1.36-1.53)	0.60	0.50	0.50	0.50
	VF69	1.36 (1.28-1.44)	0.80	0.50	0.50	0.50
Brackish	M7	1.27 (1.18-1.36)	0.70	1.0	1.5	1.5
	M13	1.25 (1.16-1.35)	0.75	1.0	1.0	2.0
	M48	0.658 (0.586-0.728)	0.80	0.50	0.50	0.50
	M63	0.994 (0.927-1.07)	0.40	0.50	0.50	0.50
	M66	0.831 (0.754-0.906)	0.40	0.50	0.25	0.50

Despite intraspecific (among genotype) variability, life-history responses did not show a pattern concordant with the acute tolerance to NaCl previously observed. Indeed, no significant differences were found between salinity-tolerant and salinity-sensitive genotypes for reproductive EC$_{50}$ (one-way ANOVA: F = 0.52; d.f. = 1, 8; p = 0.493), decrease in growth (one-way ANOVA: F = 0.05; d.f. = 1, 8; p = 0.832), decrease in survival (one-way ANOVA: F = 0.61; d.f. = 1, 8; $p = 0.457$) and decrease in fitness (one-way ANOVA: F = 0.57; d.f. = 1, 8; $p = 0.473$). The only exception was the decrease in fecundity (one-way ANOVA: F = 0.65; d.f. = 1, 8; $p = 0.034$), which was steeper in brackish genotypes. Strangely, this result suggests that the production of neonates in salinity-tolerant clones exposed to increasing NaCl concentrations was more severely affected than in salinity-sensitive clones.

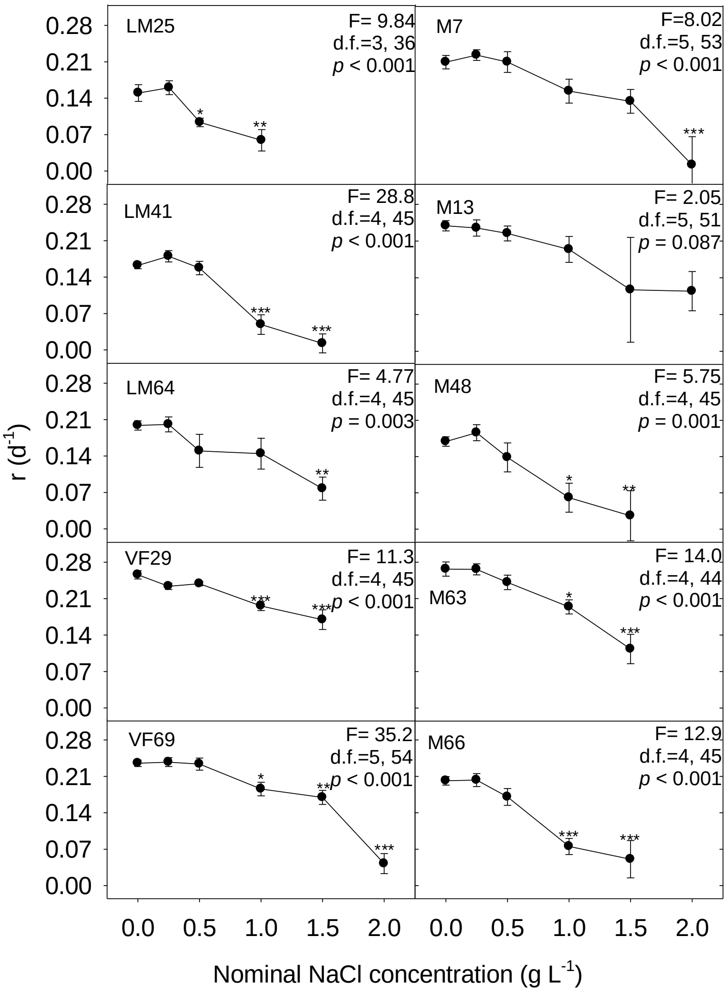

Figure 2 – Mean rate of population increase (*r*, expressed in d^{-1}) of *S. vetulus* genotypes exposed to increasing concentrations of sodium chloride (NaCl). Error bars represent standard error, and asterisks assign differences between nominal NaCl concentrations and negative control (one-way ANOVA followed by Dunnet test; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). Left and right panels represent freshwater and brackish genotypes, respectively.

Most chronic halotolerance measures were also not related to previous acute halotolerance measures (survival time, ST; acute EC_{50}). Again, a noticeable exception to this was the negatively significant association between the decrease in fecundity and ST (Figure 4). This means that the clones that were previously more resilient to acute (lethal) saline stress were found to be the most affected by chronic saline stress, in terms of their fecundity. This contradictory result is in agreement with the difference observed in the decrease in fecundity between brackish and freshwater clones (see above), since brackish clones had obtained higher ST values. Although weaker, a positive association was also found between acute EC50 and the decrease in growth and the decrease in survival (Figure 5). This demonstrates that clones that were previously more resistant to acute saline stress were also more resistant to chronic saline stress in terms of growth and survival, contrarily to what was found for fecundity and ST.

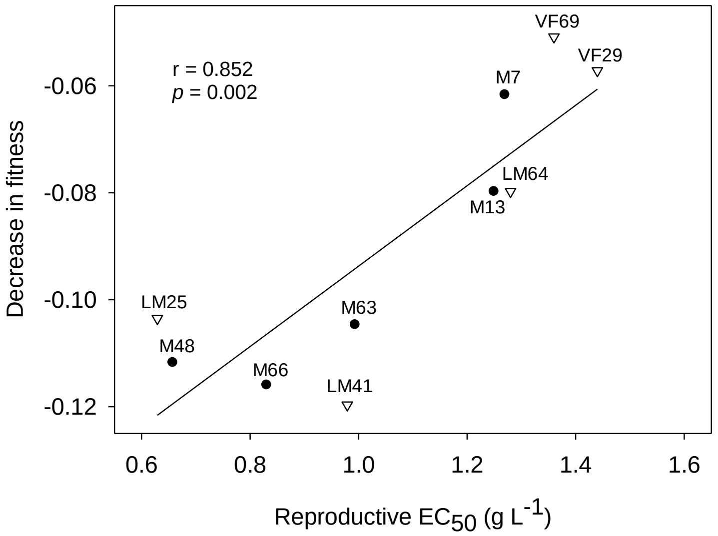

Figure 3 – Relative halotolerance of *S. vetulus* genotypes: association between the decrease in fitness (*r*) *versus* reproductive EC_{50} values. Black circles and white triangles represent brackish and freshwater genotypes, respectively.

PCA ordination (Figure 5) confirmed previous findings that most endpoints were associated with acute EC_{50} data and did not discriminate between populations. This can be observed in PCA dimension 1, where no effects were found between salinity-tolerant and salinity-sensitive clones (one-way ANOVA on PCA scores: d.f. = 1, 8; MS = 110; F = 1.24; p = 0.299); inded, brackish and freshwater clones occurred on both sides of the diagram. On the contrary, significant effects between salinity-tolerant and salinity-sensitive clones were found for PCA dimension 2 (one-way ANOVA on PCA scores: d.f. = 1, 8; MS

= 337; F = 6.34; p = 0.036), as seen by the segregation of genotypes between the top (freshwater) and bottom (brackish) panels. Confirming what was observed previously, this exceptional segregation was built on the basis of decrease in fecundity (see arrow direction), which was inversely related to ST (Figure 6).

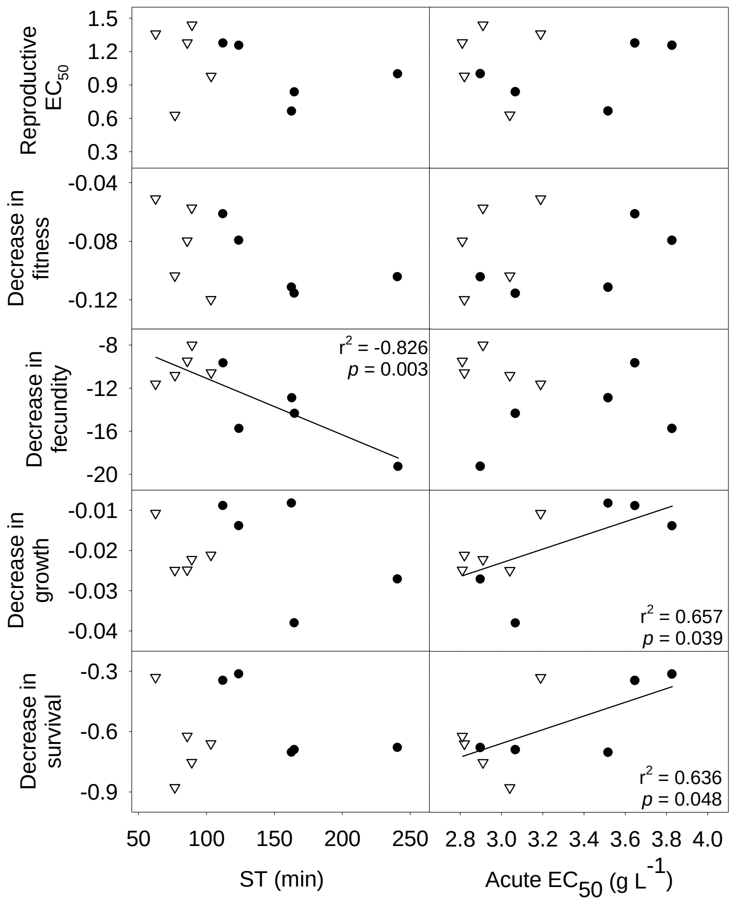

Figure 4 – Relative halotolerance of *S. vetulus* genotypes: relationship between chronic halotolerance measures (this study) and acute halotolerance measures (from Loureiro et al., 2012). ST stands for survival time. Black circles and white triangles represent brackish and freshwater genotypes, respectively.

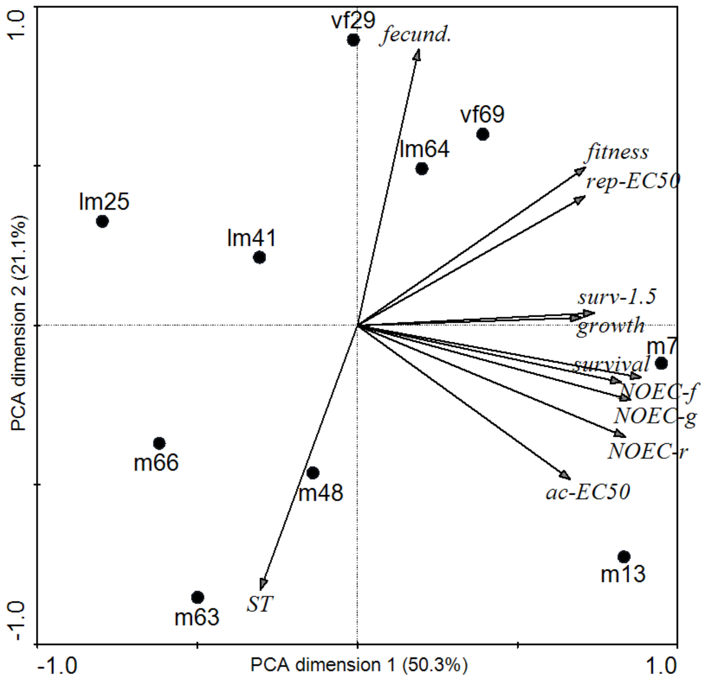

Figure 5 – Principal components analysis (PCA) biplot of acute and chronic halotolerance data for the studied *S. vetulus* genotypes. Genotypes are spatially positioned according to their relative sensitivity to NaCl, and the contribution of each endpoint is given by the direction and length of the corresponding arrow.

DISCUSSION

This study revealed intra-specific (i.e. clonal) variability in the life history responses of *S. vetulus* populations, but no evidence that clones that were previously identified as being more halotolerant were best suited to cope with salinity. Although these conclusions stem from experiments with a low number of clones, they were selected from a larger set of genotypes (Loureiro et al., 2012) as being among the most tolerant (brackish) and the most sensitive (freshwater) ones. Although contradictory, the results reflect the different responses provided by each parameter. Loureiro et al. (2012) demonstrated that the standard EC_{50} endpoint was uncorrelated with the survival time (ST). Most life history parameters (this study) were associated with the acute EC_{50}, while fecundity was inversely related with ST (see Figure 5). The decrease in fitness and the reproductive EC_{50} were not associated with either of the acute halotolerant measures (see Figures 4 and 5). This seems to suggest a weak differentiation among clones in terms of halotolerance, as previously discussed by Loureiro et al. (2012). Indeed, the only evidence supporting the higher resilience of brackish clones to salinity is their higher ability to survive to extremely lethal levels of NaCl (measured as survival time, ST, at 6 g L^{-1}), as reported in Loureiro et al. (2012).

Overall, most parameters indicated that clones M7, M13 (brackish), and VF69 (freshwater) were the most resilient to sublethal levels of NaCl (Figure 5), but no consistent pattern was observed in terms of the previously documented acute halotolerance of the full set of genotypes. However, the decrease in fecundity was not concordant with this, and, as a result, the most resilient genotypes to sublethal levels of NaCl were VF29, VF69, and LM64 (all freshwater clones), in terms of fecundity. This produced the only difference that was found between genotypes with different environmental backgrounds (brackish vs. freshwater). However, this was not reflected in fitness, because this parameter incorporates fecundity and survival, as well as the timing of reproduction and death. Nonetheless, the fact that the reproductive output of brackish clones was more affected than in freshwater clones must have a biological meaning.

One possible explanation for the steeper decrease in fecundity of brackish clones under sublethal NaCl levels is a trade-off mechanism. Let us assume that brackish genotypes are best suited to endure extremely lethal saline stress (> acute EC_{50}), as demonstrated by ST (Loureiro et al., 2012), even though this difference is not overwhelming (ST varies from 63-103 min to 113-241 min, for freshwater and brackish clones, respectively). Bearing in mind such differences are genetically-determined, this means that brackish clones possess some feature(s) that allows them to endure saline stress, at least on the short-term. Most probably, this feature is related to physiological differences in their ability to coping with osmotic pressure; we will later return to this issue. The cost of bearing this physiological adaptation could imply less energy available for reproduction under saline stress, provided two conditions are verified: a) this physiological trait is expressed in the presence of NaCl, starting at sublethal salinity levels; b) the expression of the trait is energetically costly. Trade-offs between energy allocated to growth *versus* energy allocated to reproduction have also been investigated in *Daphnia* exposed to predator chemical cues (e.g. Weber & Declerck, 1997; Declerck & Weber, 2003).

Despite the evidences that *S. vetulus* brackish clones do belong to a physiologically distinct race (*sensu* Weider & Hebert, 1987) from freshwater clones, this did not translate onto differences in fitness. Therefore, the selective forces from the environment of origin were not powerful enough to elicit a robust local adaptation pattern. This is contrary to what has been found in *D. pulex* (Weider & Hebert, 1987), *D. magna* (Teschner, 1995), and *D. thomsoni* (Barry et al., 2005). In the first two examples, the strength of local forces (salinity) was so high that some brackish genotypes performed worse under freshwater conditions, when compared to brackish conditions. This was viewed as a loss of their original freshwater adaptation (Teschner, 1995). However, unlike *Simocephalus* and other *Daphnia* (e.g. *D. longispina* group), these taxa can tolerate intermediate levels of salinity (up to 13), probably by exhibiting a hyper-osmoconformer physiological response (as shown in *D. exilis*; see Heine-Fuster et al., 2010).

Freshwater cladocerans perform hyperosmotic regulation of haemolymph (Pennak, 1985; Aladin, 1991; Aladin & Potts, 1995), by retaining salts consumed with food and absorbing salt from the external medium in the maxillary gland (adults) and nuchal organ (embryos) (Aladin, 1991). On the contrary, marine cladocerans perform hypoosmotic regulation of haemolymph, while some species may exhibit both mechanisms in brackish conditions (Aladin, 1991). Most cladocerans species seem to be stenohaline, becoming passive osmoconformers after a certain threshold; field and laboratory data suggest this happens at < 2 salinities (Schallenberg et al., 2003; Sarma et al., 2006). The extent to which freshwater cladocerans are able to cope with high internal osmolarities seems to define their tolerance to salinity. This tolerance seems to be very limited in *S. vetulus*, and this is reflected in the abrupt transition from salinity levels at which organisms are healthy and reproducing to lethal levels – a so-called "all-or-nothing-effect" (Gonçalves et al., 2007; Loureiro et al., 2012).

Such a drastic effect at low concentrations could impair the role of salinity as a local selection agent. Thus, the almost inexistent differentiation pattern between salinity-tolerant and salinity-sensistive genotypes could be due to the reduced window upon which microevolutionary forces act. Indeed, *Simocephalus* sp. populations, as well as other daphniids (e.g. *D. longispina* group), are so promptly eradicated at the onset of salinization that it was difficult for us to find them in this type of habitats. Also, this is probably why there are no studies, to our knowledge, that show a genetic differentiation pattern in brackish vs. freshwater population, in these stenohaline taxa. An alternative hypothesis to this is that the population from which the brackish clones were isolated (Lagoa de Melides) was either too young or a sporadic apparition; this would explain the lack of a strong local adaptation pattern. However, *S. vetulus* was abundant in vegetated areas of the brackish lake and ephippia were found in the sediments. This suggests the population is likely to be cyclically present. Resting eggs were also found in a neighboring brackish lake (Lagoa de Santo André). Unfortunately, we were not able to use this population as an additional replicate in this series of studies, because we could not hatch them in the laboratory.

Our study confirmed that minor salinity increases produce large impacts in stenohaline cladocerans, by causing reproductive impairment at NaCl concentrations from 1 g L^{-1} onwards and substantial mortality above 1.5 g L^{-1}. This confirms data available in existing literature (Jeppesen et al., 1994; Schallenberg et al., 2003; Marques et al., 2006; Sarma et al., 2006; Santangelo et al., 2008). Small differences were found between tolerant and less tolerant clones, but no evidence was found supporting higher fitness of brackish clones under brackish conditions. Indeed, some freshwater clones (VF29 and VF69) performed well up to NaCl concentrations of 1.5 g L^{-1}. This demonstrates that freshwater populations have the potential to resist to saline intrusion of freshwaters, by accommodating slightly tolerant genotypes within the population. Although these differences between tolerant and less tolerant genotypes are small, they may be important in competition scenarios (Lowe et al., 2007). Hence, it is likely that, at the salinity levels tested here in life history experiments, the genotype and species sorting in colonization or recolonization events produce different competitive outcomes, thus affecting clonal composition, as well as distribution and abundance patterns, in freshwater lakes or water bodies facing salinization.

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Capítulo III

Interactive effects of salinity and temperature in a progressive acclimation scenario of experimental *Daphnia galeata* populations

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O presente capítulo constitui um manuscrito em preparação para publicação em revista internacional com arbitragem científica e é, por esse motivo, apresentado na língua inglesa.

Interactive effects of salinity and temperature in a progressive acclimation scenario of experimental *Daphnia galeata* populations

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ABSTRACT

Climate change is an important source of environmental change, giving rise to interacting environmental stress scenarios (e.g. temperature rise and saline intrusion in freshwaters). However, the impacts of such interactions on natural populations must take into consideration the ability of organisms to adapt to such changes. The phenotypic adaptability of *Daphnia galeata* to salinity and temperature was investigated in this study, by evaluating *Daphnia*'s sensitivity/tolerance to salinity, at two different temperatures, along a multigenerational acclimation scenario. To do so, daphniids were acclimated to different temperatures (20ºC and 25ºC) and salinities (0 g/L and 1 g/L, using NaCl as a proxy) in an orthogonal design. The objective was to understand whether pre-adaptation to environmental stress influenced the response to the latter exposure to these stressors. The null hypothesis was that culture conditions (acclimation regimes) bear no influence on the halotolerance of the laboratorial population. The alternative hypothesis was that acclimation to culture conditions (temperature, salinity, or both) elicits an adaptive response of daphniids to saline stress or its interaction with temperature. During the acclimation period, acute (survival time) and chronic (decrease in juvenile growth) halotolerance measures were obtained at discrete timings (generations F1, F3 and F9). Acute and chronic halotolerance measures were generally unaffected by background conditions (temperature and salinity regimes). In fact, exposure temperature was the main determinant of the acute and chronic toxicity of NaCl: daphniid sensitivity (measured as the decrease of survival time or juvenile growth) was consistently higher at the highest temperature, irrespective of the background conditions. This temperature-dependent effect of temperature was nullified after nine generations, but only when animals were acclimated to both stressors (salinity and higher temperature). This is a complex interaction scenario, which must be taken into consideration in extrapolations and predictions for climate change.

KEYWORDS

Phenotypic plasticity; *Daphnia galeata*; salinity-temperature interactions; acclimation

INTRODUCTION

Fluctuations in environmental conditions are responsible for environmental stress in ecosystems, modulating structure and abundance dynamics of natural populations (Bijlsma & Loeschcke, 2005) and, consequently, leading to significant impacts on ecological processes (Boersma et al., 1999; Bijlsma & Loeschcke, 2005). In fact, environmental stress is considered a driver in the adaptation and evolution of populations to changing environments (Bijlsma & Loeschcke, 2005; Van Doorslaer et al., 2009a). Therefore, the success of natural populations is dependent on their ability to cope with new environmental conditions (Bijlsma & Loeschcke, 2005; Van Doorslaer et al., 2009a), by changing their behavior, life history and physiology responses (Weber & Declerck, 1997; Boersma et al., 1998; Bijlsma & Loeschcke, 2005; Castro et al., 2007; Van Doorslaer et al., 2009a). On the short-term, organisms adapt via acclimation to new environmental conditions – phenotypic plasticity (Bijlsma & Loeschcke, 2005; Castro et al., 2007). On the longer term, natural selection acts on genotypes by selecting the fittest ones under the new environmental conditions – local adaptation (De Meester, 1996a, 1996b; Cousyn et al., 2001; Van Doorslaer et al., 2007; Van Doorslaer et al., 2009a). Thus, the impacts of environmental change on natural populations must be investigated taking into account the organisms' ability to adapt to such change.

Climate change is an important source of environmental change, because it is considered a threat to biodiversity (IPCC, 2002, 2008), and freshwater resources are particularly vulnerable to climate change (IPCC, 2008). Coastal freshwater ecosystems are potentially subject to saline intrusion according to predictions of sea level rise and observation of coastal erosion (IPCC, 2008; Nielsen & Brock, 2009; Loureiro et al., 2012). Salinity effects in freshwater ecosystems has received considerable attention, because it is a selective pressure with implications in the structure and dynamic of populations (e.g. Schallenberg et al., 2003; Sarma et al., 2006; Gonçalves et al., 2007; Nielsen et al., 2008;

Loureiro et al., 2012) and species richness (e.g. Hall & Burns, 2002; Nielsen et al., 2008; Brucet et al., 2010; Duchet et al., 2010). Global warming can also promote salinisation of freshwaters (Chopelet et al., 2008; IPCC, 2008), because higher temperatures can lead to increased evaporation, extended droughts and reduced rainfall (IPCC, 2008; Nielsen & Brock, 2009). On the other hand, changes in temperature alone could modify the physiological responses of organisms, in terms of growth, metabolic rate and life history (Chopelet et al., 2008; Van Doorslaer et al., 2009b; Chen & Stillman, 2012). Several studies reported that temperature is a main factor affecting community structure (Van Doorslaer et al., 2009a; Kaya et al., 2010; Benincà et al., 2011) and ecosystem processes (Brown et al., 2004; Dang et al., 2009). Temperature increase and salinisation may therefore co-occur as stressors in vulnerable coastal freshwater ecosystems.

The interactive effects between salinity and temperature in freshwater organisms have received the attention of several authors, particularly for zooplankton (e.g. Hall & Burns, 2002; Brucet et al., 2009; Kaya et al., 2010; Chen & Stillman, 2012). Zooplankters play a key role in the maintenance of the ecosystem dynamics, as grazers and nutrient cyclers (Jeppesen et al., 1997; Lampert, 2006; Jeppesen et al., 2007; Van Doorslaer et al., 2009a). Therefore, it is important to understand their ability to survive and reproduce under simultaneous stressors. In fact, several authors reported that interactive effects of stressors compromises trophic interactions, thus compromising ecosystem functioning (e.g. Hall & Burns, 2002; Winder & Schindler, 2004; Brucet et al., 2010; Chen & Stillman, 2012). However, this is dependent of the degree of environmental variability and the long-term effects of acclimation (Chen & Stillman, 2012). There are still insufficient studies that consider pre-adaptation or acclimation in the sensitivity or resilience of organisms/populations to the interactive effects of simultaneous stressors (see e.g. Chen & Stillman, 2012).

Bearing this in mind, the present study intends to investigate the phenotypic component in the adaptability to salinity and temperature, and their interactive effects, in an experimental population of *Daphnia galeata*. Our objective is to assess whether the acclimation of organisms to different temperature (20ºC and 25ºC) and salinity (0 g/L and 1 g/L, using NaCl as a proxy) regimes influences their sensitivity to salinity at two different exposure temperatures (20ºC and 25ºC). The null hypothesis is that culture conditions (acclimation regimes) bear no influence on the halotolerance of the laboratorial population. The alternative hypothesis is that acclimation to culture conditions (temperature, salinity, or both) elicits an adaptive response of daphniids to saline stress or its interaction with temperature. Because previous data (Loureiro et al., 2012; Loureiro et al., in press) suggested acute and chronic responses to NaCl were not coupled, we will examine survival time and juvenile growth as measures of halotolerance. A progressive acclimation scenario will be used, examining haloterance at distinct post-acclimation moments (after 1, 3, and 9 generations).

MATERIAL AND METHODS

Cultures and test organisms

For this study, four cultures of *Daphnia galeata* were established from an existing culture (clone B, from our laboratory), and reared in moderately hard reconstituted water (123 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 96 mg L^{-1} of $NaHCO_3$, 60 mg L^{-1} $CaSO_4 \cdot 2H_2O$, e 4 mg L^{-1} KCl), supplemented with 4 mL L^{-1} of a standard organic additive (algal extract) and vitamins. This initial culture was maintained under a temperature of 20±2ºC and a $16h^{L}:8h^{D}$ photoperiod, and organisms were fed three times a week (Monday, Wednesday, Friday) with a *Pseudokirchneriella subcapitata* ration of 1.5×10^5 cells mL^{-1}. For further details on daphniid culture medium and procedures, algal cultures, see Castro et al. (2007), Gonçalves et al. (2007), and Loureiro et al. (2012).

Each new culture, derived from the initial culture (see above), was submitted to one of four different combinations of temperature and salinity: 0 g L^{-1} of NaCl at 20ºC; 1 g L^{-1} of NaCl at 20ºC; 0 g L^{-1} of NaCl at 25ºC; and 1 g L^{-1} of NaCl at 25ºC. Neonates from the initial culture (see above) were used as propagules (F_0 generation) to inoculate each new culture. All cultures were reared under the same conditions of the initial culture, except in terms of temperature and NaCl concentration. These conditions were maintained for nine generations, promoting the acclimation of organisms to the new laboratorial conditions. Neonates from F_1, F_3 and F_9 (born from F_0, F_2 and F_8 mothers, respectively), from the four cultures, were used for halotolerance tests (see below) at two exposure temperatures (20ºC and 25ºC). To assure homogeneous quality and standardisation, culture renewal and tests were always performed with neonates (less than 24 h old) born between the 3rd and 5th broods of each F_x mothers.

Survival time trials (acute halotolerance)

Survival time trials (ST) were adapted from Ribeiro et al. (2000) and Lopes et al. (2005) and followed the same procedures described by Loureiro et al. (2012). ST was used as an acute measure of halotolerance, since it is more advantageous and sensitive than the standard acute EC_{50} (Loureiro et al., 2012). It was measured as the time to immobilisation of a batch of neonates individually exposed to 6.0 g L^{-1} NaCl (prepared in moderately hard reconstituted water). These trials were performed at 20ºC and 25ºC, using 9-12 individuals per temperature from the same batch of neonates; neonates were individually placed in 2 mL of test solution in 24-well culture plates. Experiments were repeated 5 times, with each replicate using a batch of organisms produced in different days or from different culture flasks (randomised block design). Organisms were observed every 15 min from the beginning of the trial until 180 min, every 30 min from 180 min to 360 min, every 1 h after 360 min. Organisms were scored as immobile under the stereoscope (8-10x magnification) if they were not able to swim after gentle and repeated prodding. Trials ended when all organisms were considered as immobilised in two consecutive observations. In all survival time trials, controls (neonates exposed to unspiked culture medium) were performed, following the same procedures; trials were valid only if no immobilised individuals were recorded in the control at the end of the test.

To normalise the degrees of freedom between halotolerance measures (see also below), we calculated the mean ST for each replicate (thus averaging 9-12 individual observations). This also allowed minimising some noise (individual variability) in the data (see also Loureiro et al., 2012).

Juvenile growth assays (chronic halotolerance)

Juvenile growth rate was selected as a chronic halotolerance measure, because it is viewed as a good estimate of fitness (Lampert & Trubetskova, 1996; Chopelet et al., 2008). Juvenile growth tests were performed by exposing neonates to a sequence of NaCl concentrations (0.00, 0.75 and 1.5 g L^{-1}), for 3 d. Test solutions (25 mL) were obtained by dissolving NaCl in culture medium (see above). Growth assays were performed at 20ºC and 25ºC, using 7-10 individuals per treatment (1 individual per flask) from the same batch of neonates. Assays were repeated 3 times, with each replicate using a batch of organisms produced in different days or from different culture flasks (randomised block design). Test solutions were renewed once (at day 2) and organisms were fed daily with 0.75×10^5 cells mL^{-1} of *P. subcapitata*. At the end of the test period, all organisms were measured and the overall biomass was estimated for each NaCl concentration:

$\ln w = 1.468 + 2.83 \cdot \overline{\ln L}$ (from Bottrell et al., 1976),

where ln w is the natural logarithm of dry weight (in µg) and $\overline{\ln L}$ is the geometric mean length (mm) of the individuals in each treatment (McCauley, 1984). Juvenile growth rates (g_j) were calculated from biomass data:

$g_\text{j} = (\ln w_\text{f} - \ln w_\text{i}) / \Delta\text{t}$ (following Lampert & Trubetskova, 1996),

where w_f is the final biomass (on day 3, in µg), w_i is the initial biomass (on day 0, in µg), and Δt is the time interval (3 days). The initial biomass (w_i) was determined in a subsample (n = 10) of neonates in the beginning of the experiments.

We calculated the decrease in juvenile growth rate (Δg_j) as a halotolerance measure, for each replicate assay (block). This was done by expressing Δg_j as the slope of the regression line of g_j as a function of NaCl concentration (see similar approach by Loureiro et al., in press). In this way, a single halotolerance measure was extracted from the three tested concentrations, thus producing a similar scenario to ST.

Statistical analyses

To test for differences in ST and Δg_j among different culture conditions (acclimation regimes), we used two-way ANOVAs, using background (culture conditions) and exposure temperatures as fixed factors. Tukey tests were performed to tease out differences among backgrounds. When a significant background x interaction was observed, simple main effects were used to explore the differences between temperatures at each level of the factor background (Quinn & Keough, 2002). Analyses were performed separately for each generation (F_1, F_3, F_9). ST values were ln-transformed prior to the analyses, to correct for non-normality. A fixed significant level of 0.05 was used in all analyses, which were performed with Minitab software (Minitab Inc., USA).

RESULTS

Acute and chronic halotolerance measures responded slightly differently to background conditions (temperature and salinity regimes) and exposure temperature. First, we will analyse the juvenile growth rate (chronic halotolerance). For generations F1 and F3, the decrease in juvenile growth rate was consistently higher (i.e. lower Δg_j) in the highest temperature (25ºC), irrespectively of the background conditions (Figure 1 and Table 1). Also, background conditions did not exert a significant effect on the chronic halotolerance of *D. galeata* (Table 1). This means that the culture temperature and salinity conditions were unimportant as determinants of the *D. galeata* halotolerance, while, on the contrary, exposure temperature was a major determinant of the halotolerance. Indeed, organisms were more affected by NaCl at 25ºC than at 20ºC. This scenario was slightly altered in the F9 generation: a marginally significant interaction between background conditions and exposure temperature was observed (Figure 1 and Table 1). This happened because the effect of the exposure temperature on Δg_j was significant for all background conditions (simple main effects analysis: 0 g L^{-1} of NaCl at 20ºC, $p = 0.006$; 1 g L^{-1} of NaCl at 20ºC, $p = 0.004$; 0 g L^{-1} of NaCl at 25ºC, $p < 0.001$), except one (1 g L^{-1} of NaCl at 25ºC, $p = 0.57$). These evidences demonstrate that the simultaneous pre-conditioning to both stressors (salinity and temperature) eliminated the effect of temperature on the chronic halotolerance of *D. galeata*, after nine generations.

Similarly to chronic halotolerance, the major determinant of acute halotolerance was exposure temperature (Table 2), with lower survival time at 6 g L^{-1} of NaCl being recorded at 25ºC (Figure 2). This was irrespective of the background conditions (no significant background x temperature interaction), for generations F1 and F3. Unlike for Δg_j, background conditions also significantly affected ln(ST); overall, this was due to the culture under standard (unstressed) conditions (0 g L^{-1} of NaCl at 20ºC), which revealed an overall lower ln(ST) (Tukey tests, $p < 0.05$). In the F9 generation, however, a significant interaction between background conditions and exposure temperature was observed (Figure 2 and Table 2). This happened because the effect of the exposure temperature on ln(ST) was significant for all background conditions (0 g L^{-1} of NaCl at 20ºC: $p < 0.001$; 1 g L^{-1} of NaCl at 20ºC: $p < 0.001$; 0 g L^{-1} of NaCl at 25ºC: $p < 0.001$), except the one where both stressors were combined (1 g L^{-1} of NaCl at 25ºC: $p = 0.11$). This is similar to what was found for chronic halotolerance.

Table 1 – Two-way ANOVA summary table of the effects of background, temperature and their interaction on the decrease of juvenile growth (Δgrowth) in *Daphnia galeata*, along a progressive acclimation scenario (generations F1, F3, F9).

	Source	df	Adj MS	F	*p*
Δgrowth F1	Background	3	0.000694	0.56	0.65
	Temperature	1	0.072540	58.0	<0.001
	Background x temperature	3	0.000870	0.70	0.57
	Error	16	0.001250		
Δgrowth F3	Background	3	0.002118	1.59	0.23
	Temperature	1	0.099775	74.9	<0.001
	Background x temperature	3	0.001471	1.10	0.38
	Error	16	0.001332		
Δgrowth F9	Background	3	0.002573	2.76	0.08
	Temperature	1	0.033716	36.2	<0.001
	Background x temperature	3	0.002966	3.18	0.05
	Error	16	0.000932		

Table 2 – Two-way ANOVA summary table of the effects of background, temperature and their interaction on the survival time (ST) in *Daphnia galeata*, along a progressive acclimation scenario (generations F1, F3, F9).

	Source	df	Adj MS	F	*p*
ln(ST) F1	Background	3	0.10593	4.36	0.01
	Temperature	1	0.23709	9.76	0.004
	Background x temperature	3	0.03694	1.52	0.23
	Error	32	0.02430		
ln(ST) F3	Background	3	0.09413	3.17	0.04
	Temperature	1	0.15308	5.15	0.03
	Background x temperature	3	0.05333	1.79	0.17
	Error	32	0.02972		
ln(ST) F9	Background	3	0.22623	14.5	<0.001
	Temperature	1	0.54566	35.0	<0.001
	Background x temperature	3	0.14948	9.59	<0.001
	Error	32	0.01559		

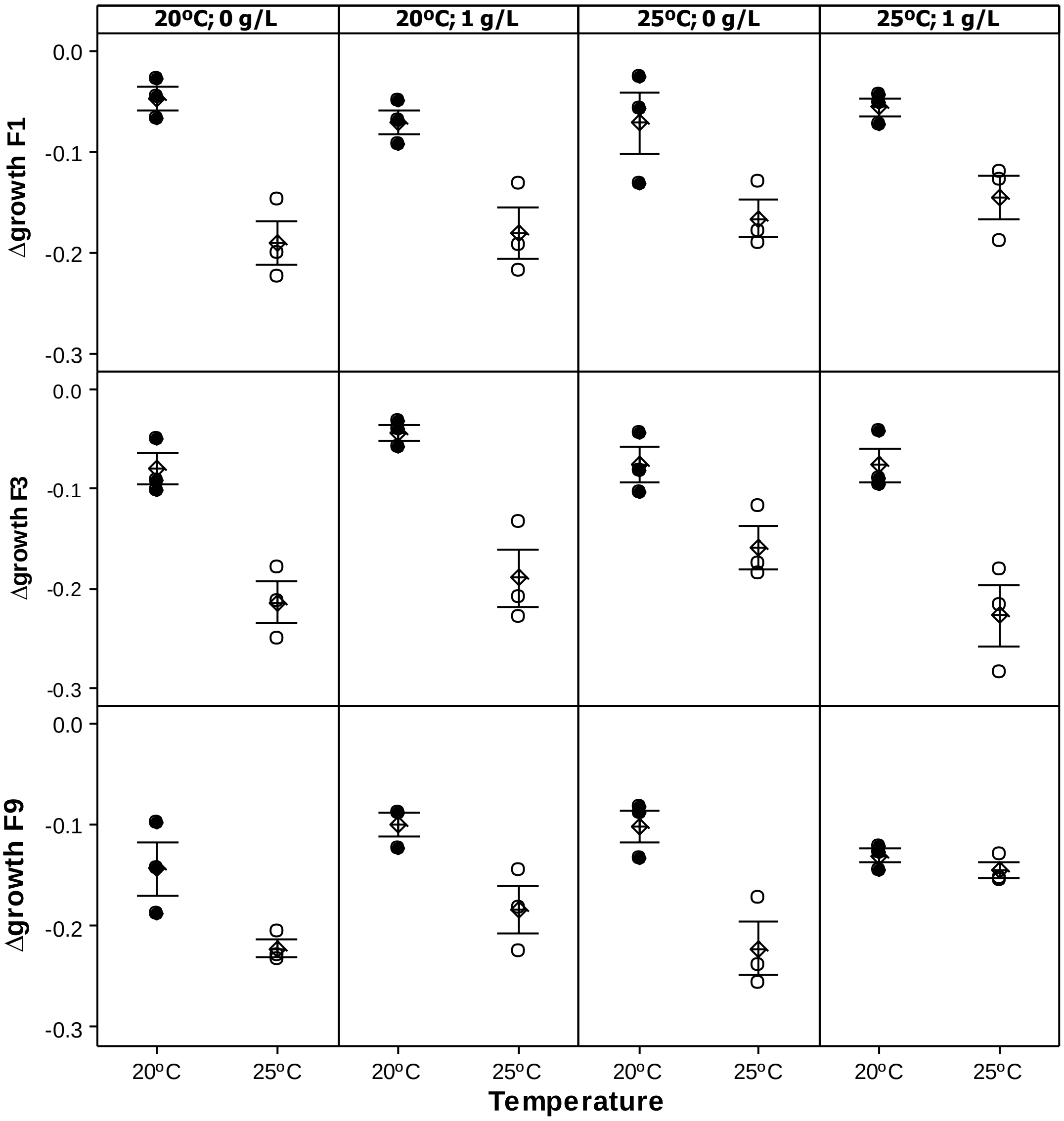

Figure 1 – Chronic halotolerance (expressed as the decrease in juvenile growth rate in response to a NaCl gradient) of *Daphnia galeata* reared in different backgrounds (temperature and salinity regimes – see panel legend). Each row of graphs represents a progressive acclimation scenario (generations F1, F3, and F9). Individual observations are shown for each tested temperature (closed and open circles), along with mean and SE bars.

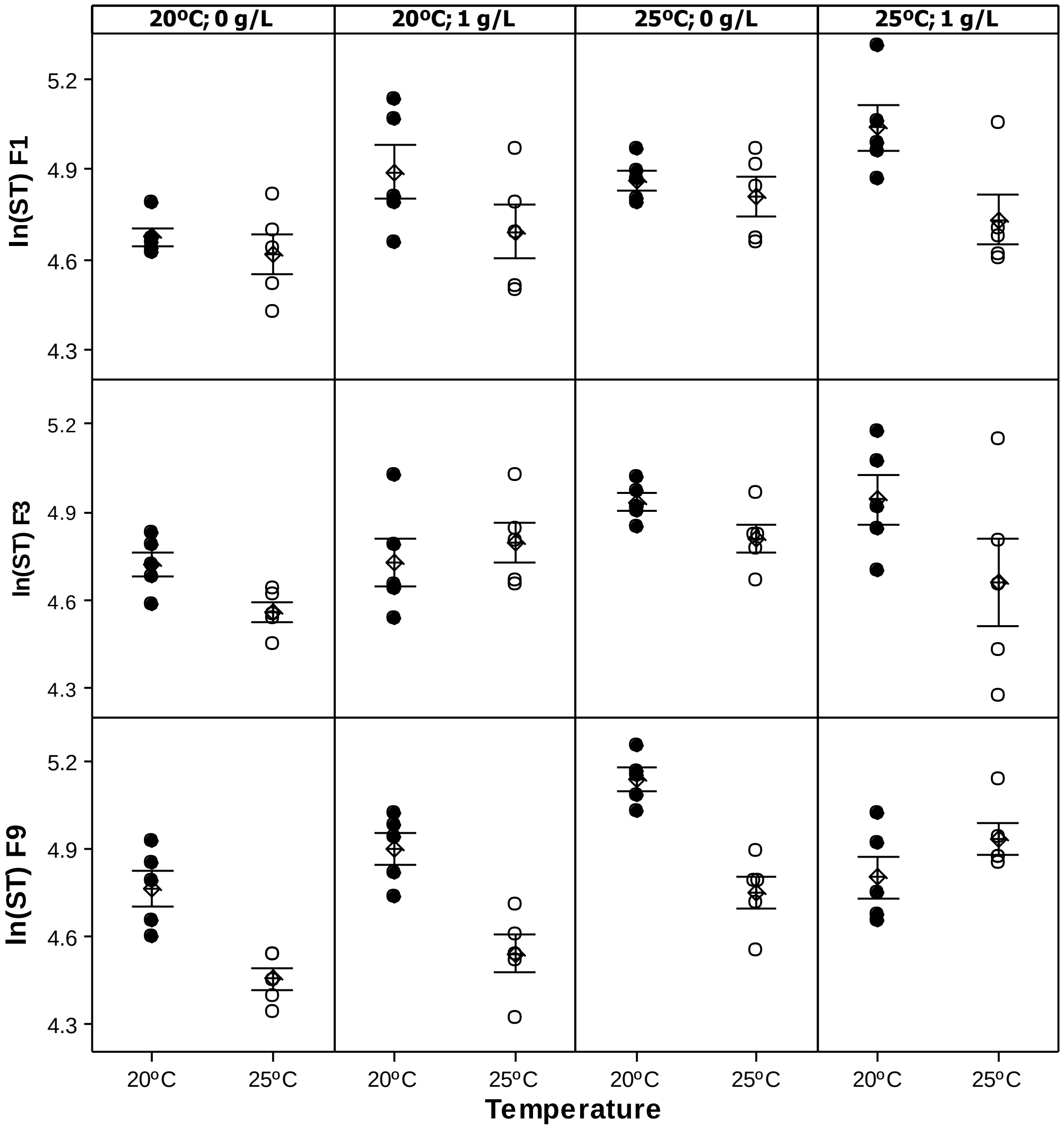

Figure 2 – Acute halotolerance (expressed as the ln-transformed survival time in response to a lethal NaCl concentration) of *Daphnia galeata* reared in different backgrounds (temperature and salinity regimes – see panel legend). Each row of graphs represents a progressive acclimation scenario (generations F1, F3, and F9). Individual observations are shown for each tested temperature (closed and open circles), along with mean and SE bars.

DISCUSSION

Acclimation to culture conditions elicited a progressive adaptive response of daphniids to the interaction between saline stress and temperature; i.e. culturing conditions did influence the halotolerance of the laboratorial population (rejection of null hypothesis). Exposure temperature was the main determinant of the acute and chronic toxicity of sodium chloride. The decrease of survival time or juvenile growth was overall consistently higher at the highest temperature, irrespective of the background conditions. However, this temperature effect was nullified after nine generations, but only when animals were acclimated in the presence of both stressors (salinity and higher temperature), and this was convincingly observed both in acute and chronic endpoints. Acclimation to salinity or temperature individually did not produce significant effects in the sensitivity of the organisms to salinity. The exception was a mild effect of background observed in acute responses (ST), where pre-exposure to either stressor slightly enhanced the halotolerance of the daphniids; overall, ST of the animals cultured in their original conditions (no NaCl, 20ºC) was lower. This effect was not very pronounced and none of these conditions affected the temperature-dependence effect over halotolerance. As stated above, this only happened when animals had been cultured in the presence of both salinity and high temperature, and only after nine generations. This is a complex interaction scenario, which must be taken into consideration in extrapolations and predictions for climate change.

The interaction of both stressors has been studied previously with cladocerans (e.g. Hall & Burns, 2002; Brucet et al., 2009; Chen & Stillman, 2012). Chen & Stillman (2012) report that *D. pulex* is responsive to acclimation, in terms of metabolic rate, generation time and acute tolerance to extreme variations of salinity and temperature, but this is dependent of the amount of environmental variability. As in our study, Chen & Stillman (2012) refer that temperature is the main determinant of tolerance to salinity and temperature. In fact, temperature variations should explain different salinity tolerance since osmoregulatory ability of some species depends on temperature (Aladin & Potts, 1995; Brucet et al., 2009). However, extreme salinity variation induced cross-susceptibility to environmental extremes in temperature and salinity (Chen & Stillman, 2012). This may indicate a connection in the responses to stress. In our study, we also observed that pre-conditioning to stressful conditions (temperature, salinity, or both) elicited a slight increase in the overall tolerance to salinity, in terms of survival time. Other authors find important to comprehend responses correlated to stress, namely in terms of resistance/coresistance (Lopes et al., 2005; Klerks et al., 2011).

The interactive effect of salinity and temperature has been shown to negatively affect the sexual reproduction of a dinoflagellate while producing a favourable effect for vegetative growth (Figueroa et al., 2011). The increase in temperature seems to be also responsible for decrease in food quality and *Daphnia* populations are susceptible to this (McFeeters & Frost, 2011); also, food quality and quantity is very important in the tolerance to other stressors, namely contaminants (e.g. Fleeger et al., 2003; Antunes et al., 2004). A larger size at maturity was reported as another response of *Daphnia* populations to warmer climate (Van Doorslaer et al., 2010). Temperature effects alone have been reported elsewhere (see above references), but ours is one of the few studies that covers the indirect effects, via pre-acclimation, of two interacting stressors. The recent work of Chen and Stillman (2012) also explores this, emphasizing the importance of incorporating acclimation/adaptation and complex environmental change scenarios (looking at interacting stressors rather than in isolation) in environmental assessments.

In general, organisms exposed to stressors have elevated metabolic demands as they face suboptimal environments, thus allocating energy for cellular or physiological responses such as osmoregulation or thermoregulation (Chen & Stillman, 2012; Hiebenthal et al., 2012). However, organisms are able to adapt to changes after few generations and this implies the allocation of energy to physiological responses, as survival and growth, and decrease metabolic rate (Haney & Wassh, 2003; Chen & Stillman, 2012). As a response to temperature increase, a possible reduction of metabolic rate in organisms could lead to reduced energy expenditure, slowing "biological time" and increasing survival, despite the imposed physiological stressor (Haney & Wassh, 2003; Guan & Wang, 2006; Chen & Stillman, 2012). Consequently, the overall resistance to toxics could increase and this permits the survival of organisms (Smolders et al., 2005; Chen & Stillman, 2012). Modifications in the tolerance limits of *Daphnia* as been demonstrated to other stressors in multigenerational studies, as metals (Bossuyt et al., 2005; Guan & Wang, 2006; Chen & Stillman, 2012). On the other hand, Duchet et al (2010) reported that it is difficult that daphnid populations are able to cope with simultaneous natural stressors (salinity and temperature) and anthropogenic stressors (larvicides). In studies where organisms are exposed to multiple stressors, without taking into account potential acclimation phenomena, effects can be overestimated. This is particularly important when addressing climate change, because of the scientific and social impacts of studies in this hot topic. Acclimation processes are important in the evaluation of the effects of contaminants in natural populations, because there is an important role of maternal effects, persisting throughout different generations (Chen & Stillman, 2012), and a redistribution of energy under stress (Guan & Wang, 2006), which can subsequently affect responses to all sorts of environmental variability.

We must emphasize that we did not look into the absolute effect of temperature or salinity, especially in terms of juvenile growth. Indeed, we found consistently higher juvenile growths under higher temperature (data not shown); however, we used the decrease of growth relatively to a control as a measure of sensitivity. Had we proceeded differently and results were more difficult to interpret, as they would translate the absolute effect of temperature as well as its impacts in the relative sensitivity. Our goal was to focus on the effect of temperature and background conditions on the sensitivity to NaCl, rather than on the temperature-dependent effects on the measured endpoints per se.

In this study, we only explored the phenotypic (immediate and multigenerational) component in the acclimation to changing environmental conditions. It is expectable, that on the long term, adaptation to changing conditions will also translate in changes in genotypes, because selection will act on extant genotypes (Weber & Declerck, 1997; Boersma et al., 1999; Van Doorslaer et al., 2009a). Indeed, studies on global warming showed that increases in temperature elicit microevolutionary processes in daphniid population dynamics (Van Doorslaer et al., 2007; Van Doorslaer et al., 2009b; Van Doorslaer et al., 2010). The impacts of climate change depend on the degree of variability in environmental fluctuations (Hedhly et al., 2008; Benincà et al., 2011; Chen & Stillman, 2012) and the adaptation of organisms allows for allele frequency alterations (Bijlsma & Loeschcke, 2005) and this could lead to local adaptation to higher temperatures (Van Doorslaer et al., 2009a). The theoretical consequences of such adaptation are numerous and may alter initial predictions of the effects of climate change, but few studies have addressed this so far; a noticeable exception is the study by Van Doorslaer et al. (2009a), which showed that local adaptation to higher temperature can reduce the establishment success of immigrant genotypes pre-adapted to warmer climate (Van Doorslaer et al., 2009a).

So, acclimation to potentially stressful conditions is very important to understand the effects of stressors in organisms and populations. Complex scenarios, such as the ones we report here – simultaneous pre-exposure to temperature and salinity nullified temperature-dependent effects on the halotolerance of daphniids – must be taken into consideration on the evaluation of the impacts of environmental change. One should not undervalue the organisms' abilities and strategies to cope with environmental fluctuations.

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Capítulo IV

Competitive outcome of *Daphnia-Simocephalus* experimental microcosms: salinity versus priority effects

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O presente capítulo constitui um manuscrito submetido para publicação em revista internacional com arbitragem científica e é, por esse motivo, apresentado na língua inglesa.

Competitive outcome of *Daphnia-Simocephalus* experimental microcosms: salinity versus priority effects

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ABSTRACT

Competition is a major driving force in freshwaters, especially given the cyclic nature and dynamics of pelagic food webs. Competition is especially important in the initial species assortment during colonization and re-colonization events, which depends strongly on the environmental context. Subtle changes, such as saline intrusion, may disrupt competitive relationships and, thus, influence community composition. Bearing this in mind, our objective was to assess whether low salinity levels (using NaCl as a proxy) alter the competitive outcome (measured as the rate of population biomass increase) of *Daphnia-Simocephalus* experimental microcosms, taking into account interactions with priority effects (sequential species arrival order). With this approach, we aimed to experimentally demonstrate a putative mechanism of differential species sorting in brackish environments or in freshwaters facing secondary salinisation. Experiments considered three NaCl situations regarding NaCl added (0.00, 0.75 and 1.50 g/L) crossed with three competition scenarios (no priority, priority of *Daphnia* over *Simocephalus,* and vice-versa). At lower NaCl concentrations (0.00 and 0.75 g/L), *Daphnia* was a significantly superior competitor, irrespective of the species inoculation order. However, the strong decrease in *Daphnia* population growth at 1.50 g/L alleviated the competitive pressure on *Simocephalus*, causing an inversion of the competitive outcome in favour of *Simocephalus*. The intensity of this inversion depended on the competition scenario. This salinity-mediated disruption of the competitive outcome demonstrates that subtle environmental changes produce indirect effects in key ecological mechanisms, thus altering community composition, which may lead to serious implications for ecosystem functioning (e.g. lake regime shifts due to reduced grazing).

KEYWORDS

Competition; priority effects; salinity; experimental microcosms; freshwater zooplankton

INTRODUCTION

The structure and abundance dynamics of biotic communities are modulated by abiotic factors, biotic relations and their interaction (Ricklefs, 2001; Bijlsma & Loeschcke, 2005; Semenchenko et al., 2007). Freshwater ecosystems, in particular, exhibit fluctuating abiotic and biotic conditions (e.g. water transparency, resource availability, predation) that influence the dynamics of species succession (Bijlsma & Loeschcke, 2005). In this matter, one of the best documented patterns in community ecology is the cyclical succession of plankton species in freshwaters (PEG model; Sommer et al., 1986), which emphasizes the interplay of abiotic factors and biotic relations. Competition plays an important role in this succession model, as a result of food limitation (Nandini et al., 2007; Adamczuk, 2010).

In fact, competition is a major driving force in freshwater systems, especially given the cyclic nature and dynamics of planktonic food webs. While intra-specific competition is important in defining equilibrium in population dynamics, inter-specific competition tends to be destabilizing, causing ecological exclusion of one or the other competitor(s) (Hutchinson, 1961; Bengtsson, 1986). Inter-specific competition generally translates into the mutual inhibition of growth rate among populations of different species that have common requirements for shared and limiting resources. Competition between populations of cladocerans can be responsible for shifts in competitor’s life-history, in terms of density, growth, juvenile survival and clutch-size (Adamczuk, 2010), leading to a co-existence scenario with different demographic cycles (Cáceres, 1998; Adamczuk, 2010).

Regulation of cladoceran community structure is modulated by colonization and re-colonization events from the ephippial egg bank (De Meester et al., 2007; Louette & De Meester, 2007). Competition is especially important in the initial species assortment (Sommer et al., 1986; Hairston, 1996; Louette et al., 2006), which depends strongly on the initial species and gene pool (producing so-called founder effects, De Meester et al., 2002; De Meester et al., 2007), as well as the environmental conditions of the system. Under such scenarios, the order at which species appear in the system may configure priority effects, in which species that appear first have a competitive advantage over latecomers (Louette & De Meester, 2007; Van Gremberghe et al., 2009). Priority effects are defined as the impact that a particular species can have on community development due to prior arrival (or hatching) at a site, and they usually result from resource and niche monopolization of early colonizers (Louette & De Meester, 2007; Loeuille & Leibold, 2008). The environmental context is known to impact the strength of priority effects or even superimpose them (e.g. Louette & De Meester, 2007). Previous experiments with *Daphnia* (Hessen, 1996; Nandini et al., 2007) have shown that the environmental context influences the competitive outcome. Louette & De Meester (2007) showed that predation may alter the competitive outcome of inter-specific relationships. Using plants as experimental subjects, several authors have shown that competitive ability or intensity is alleviated under environmental stress (e.g. Wilson & Keddy, 1986). Also, Emery et al. (2001) demonstrated that stress tolerators were consistently dominant competitors under some circumstances. The reasonable conclusion is that environmental stress, either abiogenic or biogenic, may alter radically the expected outcome of species sorting, a key process in the population dynamics of freshwater cladoceran populations (see above).

Salinity is an abiotic environmental stressor that alters the zooplankton community structure (Hall & Burns, 2002; Santangelo et al., 2008; Brucet et al., 2010; Duchet et al., 2010), even at low levels (Schallenberg et al., 2003; Sarma et al., 2006). Salinisation of freshwaters, which is a predicted consequence of global climate change and groundwater overexploitation (IPCC, 2008), represents serious implications for ecosystem functioning (e.g. promoting a lake regime shift – Jeppesen et al., 2007). At lethal salinity levels (> 2), sensitive species are purely eliminated or are unable to hatch. At lower levels, however, salinity could disrupt competitive relationships, with brackish conditions favouring different species composition than in freshwater conditions. So far, there is no experimental evidence for this in the literature.

Bearing this in mind, our objective was to assess whether low salinity levels (using NaCl as a proxy) alter the competitive outcome of a *Daphnia-Simocephalus* experimental system, taking into account interactions with priority effects (sequential species arrival order). It is expected that salinity alters the competitive outcome of inter-specific relationships, provided that there are slight differences in halotolerance between competitor species; however, it is hypothesized that priority effects (inoculation order of the competitor species) may confer some protection to the less halotolerant species. With this approach, we aim to experimentally confirm the hypothesized mechanism of differential species sorting in brackish environments or in freshwaters facing secondary salinisation.

MATERIAL AND METHODS

Cultures and test organisms

Cultures of *Simocephalus vetulus* (Müller, 1776) and *Daphnia galeata* Sars, 1864 were reared in the laboratory for several generations. Culture and test medium consisted of moderately hard reconstituted water (123 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 96 mg L^{-1} of $NaHCO_3$, 60 mg L^{-1} $CaSO_4 \cdot 2H_2O$, e 4 mg L^{-1} KCl, *sensu* ASTM (1980) and USEPA (2002)), supplemented with 4 mL L^{-1} of a standard organic additive (algal extract) and vitamins (for further details, see Loureiro et al., 2011; Loureiro et al., 2012). Reconstituted water was prepared with UV-sterile deionised water (conductivity < 10 μS cm^{-1}), obtained from mixed-bed ion exchange resins (after pre-filtration through particle filters and activated carbon cartridges). Cultures were maintained under a temperature of 20±2ºC and a $16h^L:8h^D$ photoperiod, and organisms were fed three times a week (Monday, Wednesday, Friday) with a *Pseudokirchneriella subcapitata* ration of 1.5×10^5 cells mL^{-1} (for more information on algal culture and ration, see Antunes et al., 2003; Antunes et al., 2004; Loureiro et al., 2012).

Competition experiment

The experiment was performed in transparent plastic buckets (microcosms; internal diameter: 16-19 cm; height: 21 cm) containing 4 L of test solution (three salinity levels: 0.00, 0.75, and 1.50 g of NaCl per L). Solutions were obtained by dissolving reagent-grade NaCl (salinity proxy) in the above-described culture medium. A semi-static approach was used, by renewing 50% of test medium every week; this was done by siphoning 1 L of water from each microcosm, assuring no organisms were removed, and adding 1 L of fresh medium, two times a week (Mondays and Thursdays). After medium renewal, organisms were fed with a *P. subcapitata* ration of 0.75×10^5 cells mL^{-1}. This represents a less concentrated and a less frequent algal ration than in cultures, so that food becomes somewhat more limiting towards the final stage of the competition experiment, when both competitor populations are established. All experiments were carried out under a temperature of 20±2ºC and a $16h^L:8h^D$ photoperiod. Once a week, UV-sterile deionised water was added to compensate for evaporation losses, and conductivity, pH and oxygen were measured for quality assurance criteria.

We also simulated three different competition scenarios, by manipulating priority effects (order of inoculation of competitor species). In two of the treatments, one species was introduced at day 0 and the other at day 10, thus simulating priority of *D. galeata* over *S. vetulus* (treatment D>S) and vice-versa (S>D); in the third treatment (S|D), both species were introduced at day 0 (i.e. no priority). Competition scenario (3 levels) was fully crossed with NaCl concentration (3 levels). To allow proper manipulation and visualisation, young (5-6 d old) females were used to start the experiments. All microcosms were inoculated with 10 individuals of each species, following the chronological order above. Three replicate microcosms were used per competition treatment and NaCl concentration combination.

At the end of the experiment (day 30), abundance and biomass of *Daphnia* and *Simocephalus* populations were estimated for each treatment-concentration combination. Each microcosm was filtered through a 55-µm-mesh plankton net and the corresponding filtered residue was preserved in 96% ethanol. All organisms were sorted into species and size classes (large adults, ≥ 1.8 mm; 1.2 mm ≤ small adults < 1.8 mm; juveniles, < 1.2 mm), and then counted under a stereoscope. Body length measurements (from top of head to base of caudal spine) were taken, using a stratified approach: all large adults were measured, while lengths of small adults and juveniles were measured in sub-samples of 50 individuals. Biomass estimates for both *Daphnia* and *Simocephalus* were obtained from a general length-weight relationship for daphniids (as recommended by Watkins et al., 2011):

$\ln w = 1.468 + 2.83 \cdot \overline{\ln L}$ (from Bottrell et al., 1976),

where ln w is the natural logarithm of dry weight (in µg) and $\overline{\ln L}$ is the geometric mean length (mm) of individuals in the sample (McCauley, 1984). Mean individual weight was calculated for each stratum (large adults, small adults, juveniles), and total biomass (in µg L^{-1}) was estimated taking into account the counts for each size class.

The rate of population biomass increase (r_b, in day^{-1}) was also estimated for each species, in each experimental microcosm:

$r_b = (\ln B_f - \ln B_i) / \Delta t$,

where B_f is the final population biomass (on day 30, in μg L^{-1}), B_i is the initial population biomass (on day 0 or 10, in μg L^{-1}, depending on the competition scenario), and Δt is the time interval (20 or 30 days).

Statistical analyses

The effect of salinity level on the relative composition of the experimental *Daphnia-Simocephalus* assemblage was analyzed with separate repeated-measures (RM) ANOVAs on biomass data, one for each competition scenario (D>S, S|D, S>D). Species and NaCl concentration were the within- and between-subjects factors, respectively. The natural log was used to transform biomass data and correct for non-normality. A more detailed analysis was performed on the rate of population biomass increase (r_b), because it is a more suitable estimate of competitive outcome (Louette & De Meester, 2007). Indeed, absolute abundance or biomass values on day 30 may merely reflect the fact that one or the other species were introduced first (in scenarios S>D and D>S), while r_b expresses the rate at which they grow from the starting inoculum – making it comparable between species. In this case (r_b), after running the RM ANOVA, simple main effects of species within each salinity level were analysed, using the within-cells error (from the more comprehensive RM ANOVA) term as the denominator for the *F*-test (Quinn & Keough, 2002). Since simple main effects analyses constitute a case of multiple comparisons, control over the family-wise type I error rate was performed by adjusting the significance level (α) using the Dunn–Sidak procedure ($\alpha = 0.017$). The presence of a significant interaction between species and NaCl concentration was interpreted as a salinity-induced disruption of the expected competitive outcome.

Complementarily, r_b data were analyzed with two-way ANOVAs, using NaCl concentration and species inoculation order as fixed factors, in order to explore how NaCl concentration interacted with priority effects. These analyses were run separately for each species. Whenever an interaction between NaCl concentration and species inoculation order was found, a simple main effect analysis was carried out (using the residual of the two-way ANOVA as the denominator of the F-test; Quinn & Keough, 2002) for the species inoculation order within each salinity level. For this purpose, significance level was adjusted ($\alpha = 0.017$) as described above. All other analyses used a 0.05 significance level.

RESULTS

At the end of the experiment, different relative compositions of the experimental communities were obtained (Figure 1). *Daphnia* or *Simocephalus* were overall dominant in the experimental treatments where they were given chronological advantage in the inoculation order (respectively, D>S and S>D); this was mostly noticeable in the case of *Daphnia* for the D>S treatment. When both species were inoculated at the same time (S|D), *Daphnia* was generally the dominant taxon.

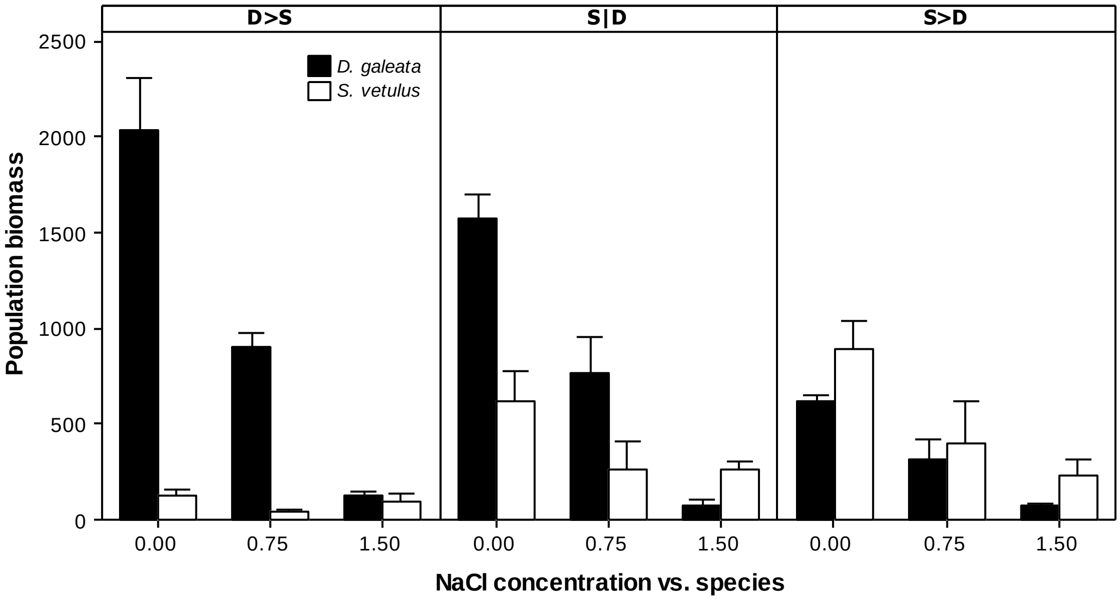

Figure 1 - Average population biomass (n = 3 experimental microcosms), in µg L^{-1}, for *Daphnia galeata* and *Simocephalus vetulus* on day 30, at three different NaCl concentrations (in g L^{-1}). Panels represent the three competition scenarios, which differ in the order of inoculation of the species (see text for codes). Error bars represent 95% confidence intervals of the mean.

However, salinity altered the relative composition of the communities in all three competition scenarios, as revealed by the significant interactions between species and NaCl concentration for all three treatments (D>S, S|D, S>D) - see Table 1. Alterations in the relative composition of the *Daphnia-Simocephalus* experimental microcosms were evident at 1.50 g L^{-1}: in S|D and S>D, salinity favoured *Simocephalus* in detriment of *Daphnia*; in D>S, the overall dominance of *Daphnia* under no salinity was nullified at this salinity level. Significant interactions between species and NaCl concentration were also found for the rate of population biomass increase (r_b; Figure 2) in the three treatments (D>S, S|D, S>D), which confirms salinity-induced disruption of the expected competitive outcome of the *Daphnia-Simocephalus* experimental microcosms - see Table 1. At 0.00 and 0.75 g L^{-1} concentrations, *Daphnia* was a significantly superior competitor than *Simocephalus* (see simple main effects, Figure 2), irrespective of the species inoculation order. At these salinity levels, priority effects thus seemed negligible, given the prevalence of *Daphnia*. However, its competitive abilities were compromised at the highest salinity level, as shown by the inversion of the competitive outcome in favour of *Simocephalus* in all competition scenarios at 1.50 g L^{-1} (Figure 2). At this concentration, priority effects influenced the degree of such inversion (Figure 2), with the most extreme case occurring when no priority was given to either species (S|D).

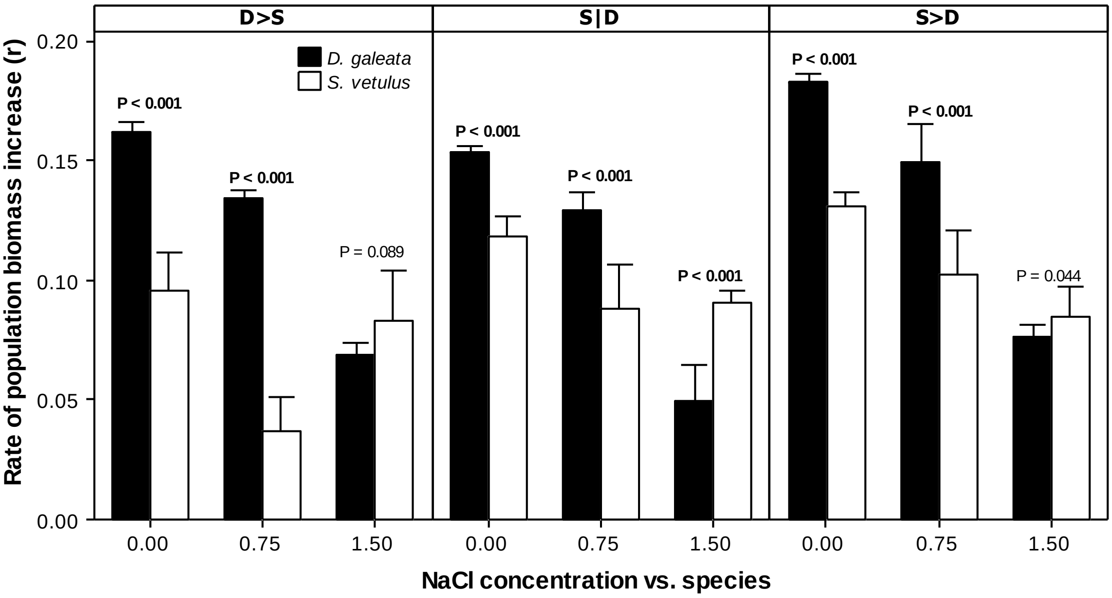

Figure 2 - Average rate of population biomass increase (n = 3 experimental microcosms), in day^{-1}, for *Daphnia galeata* and *Simocephalus vetulus* exposed to three different NaCl concentrations (in g L^{-1}). Panels represent three competition scenarios, which differ in the order of inoculation of the species (see text for codes). Error bars represent 95% confidence intervals of the mean. *P*-values are shown for pairwise differences between competitor species ($\alpha = 0.017$, adjusted for multiple comparisons) for each salinity level, within each competition scenario.

Table 1 – Summary of the RM ANOVAs applied to the ln biomass and population biomass increase rate (r_b) data, for each treatment (species inoculation order). The degrees of freedom of the numerator (df 1) and denominator (df 2) of each *F*-ratio are shown, as well as the variance (MS) of the numerator.

	Treatment	**Model**	**df 1**	**df 2**	**MS**	***F***	***P***
ln biomass	**D>S**	Between subjects					
		NaCl concentration	2	6	3.6	330.9	< 0.001
		Within subjects					
		Species	1	6	19.9	526.6	< 0.001
		Species x NaCl conc.	2	6	3.8	99.8	< 0.001
	S|D	Between subjects					
		NaCl concentration	2	6	5.9	254.8	< 0.001
		Within subjects					
		Species	1	6	0.263	5.2	0.063
		Species x NaCl conc.	2	6	2.8	55.5	< 0.001
	S>D	Between subjects					
		NaCl concentration	2	6	4.7	287.7	< 0.001
		Within subjects					
		Species	1	6	1.4	26.6	0.002
		Species x NaCl conc.	2	6	0.372	7.0	0.027
rate of increase (r_b)	**D>S**	Between subjects					
		NaCl concentration	2	6	0.005	139.4	< 0.001
		Within subjects					
		Species	1	6	0.011	140.4	< 0.001
		Species x NaCl conc.	2	6	0.005	63.8	< 0.001
	S|D	Between subjects					
		NaCl concentration	2	6	0.007	254.8	< 0.001
		Within subjects					
		Species	1	6	0.001	10.7	0.017
		Species x NaCl conc.	2	6	0.003	55.5	< 0.001
	S>D	Between subjects					
		NaCl concentration	2	6	0.009	509.9	< 0.001
		Within subjects					
		Species	1	6	0.004	52.7	< 0.001
		Species x NaCl conc.	2	6	0.002	21.9	0.002

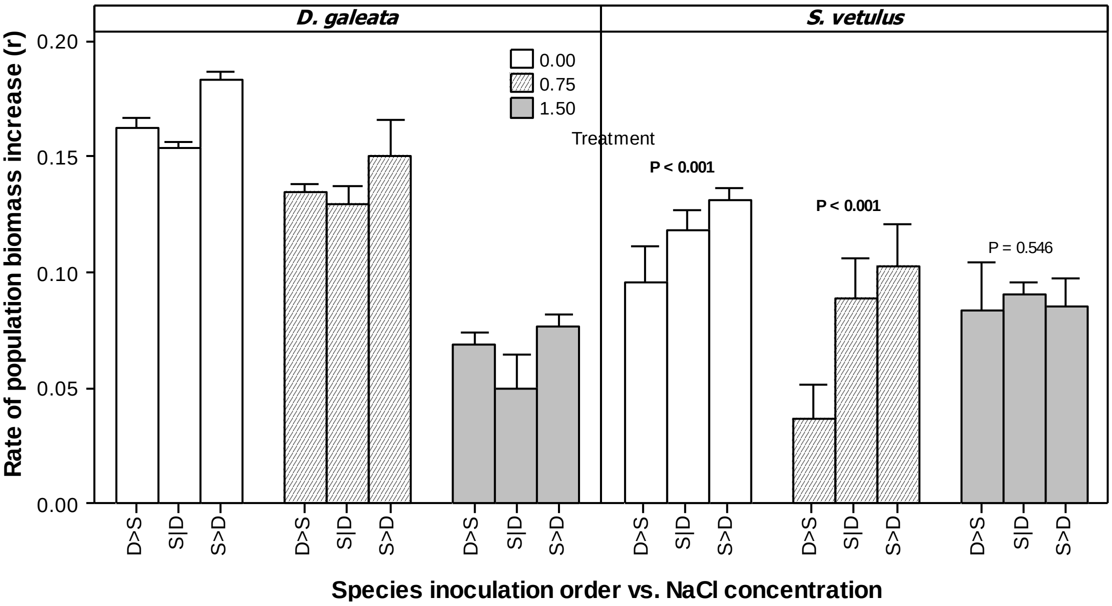

Figure 3 - Average rate of population biomass increase (n = 3 experimental microcosms), in day^{-1}, for *Daphnia galeata* (left panel) and *Simocephalus vetulus* (right panel) exposed to three NaCl concentrations (in g L^{-1}), in three competition scenarios that differ in the order of inoculation of the species (see text for codes). Error bars represent 95% confidence intervals of the mean. *P*-values are shown for differences among inoculation order ($\alpha = 0.017$, adjusted for multiple comparisons) for each salinity level, for *S. vetulus*.

In order to analyse priority effects in more detail, we plotted the data for each species separately (Figure 3). *Daphnia* biomass increase rate was mainly affected by NaCl concentration, decreasing monotonically from 0.00 to 1.50 g L^{-1}. To a lesser extent (see *F*-ratios, Table 2), *Daphnia* increase rate was also affected by the inoculation order (i.e. competition scenario), with $r_{S>D} > r_{D>S} > r_{S|D}$. Surprisingly, *Daphnia* biomass increase rate was consistently higher in the S>D treatment; this supports its superior competitive ability, showing that this species is not negatively affected (being apparently favoured) by priority effects from *Simocephalus*. Unlike for *Daphnia*, main effects of inoculation order (i.e. priority effects) were not consistent across salinity level for *Simocephalus*, as shown by the significant interaction between these two factors (Table 2). Both NaCl concentration and inoculation order were equally important as sources of variation of the *Simocephalus* biomass increase rate (see *F*-ratios). At 0.00 and 0.75 g L^{-1} NaCl, differences in *Simocephalus* population biomass increase rates were consistent, being maximum when priority was given to *Simocephalus* (S>D), intermediate when both species were inoculated at the same time (S|D), and minimum when *Daphnia* had the initial advantage (D>S). However, no significant differences were found among different inoculation orders at 1.5 g

L^{-1} NaCl. These evidences show that *Simocephalus* population growth was constrained by its superior competitor (*Daphnia*) irrespective of inoculation order, although this depressive effect was felt more intensely when priority was given to *Daphnia*. At 1.5 g L^{-1}, *Simocephalus* population biomass increased at the same rate in all competition scenarios, suggesting that the competitive pressure imposed by *Daphnia* was alleviated at this NaCl level, irrespective of the species inoculation order. This works as a salinity-mediated mechanism that causes disruption of the competitive outcome as expected in a no-salinity scenario.

Table 2 – Summary of the two-way ANOVAs applied to the population biomass increase rate (r_b) data, for each competitor species. The degrees of freedom of the numerator (df 1) and denominator (df 2) of each *F*-ratio are shown, as well as the variance (MS) of the numerator. Treatment stands for the species inoculation order.

	Taxon	**Model**	**df 1**	**df 2**	**MS**	***F***	***P***
rate of increase (r_b)	***Daphnia***	NaCl concentration	2	18	0.025	988.9	< 0.001
		Treatment	2	18	0.001	56.9	< 0.001
		NaCl conc. x treatment	4	18	6.2×10^{-5}	2.5	0.081
	Simocephalus	NaCl concentration	2	18	0.004	51.1	< 0.001
		Treatment	2	18	0.003	40.5	< 0.001
		NaCl conc. x treatment	4	18	8.3×10^{-4}	11.5	< 0.001

DISCUSSION

This experimental study demonstrated a salinity-mediated disruption of the competitive outcome in *Daphnia*-*Simocephalus* microcosms. This demonstrates that subtle environmental changes, such as low levels of salinity, produce indirect effects in key ecological mechanisms, namely species sorting. Thus, our results support the hypothesized mechanism of differential species sorting within zooplankton communities, in brackish environments or in freshwaters facing secondary salinisation. Also, this study demonstrates that salinity, even at low levels, was strong enough to nullify priority effects (see below).

Up to 0.75 g L^{-1}, *Daphnia* demonstrated to be a superior competitor, independently of the order of inoculation (see Figures 2 and 3). However, the order of inoculation was important for the inferior competitor, *Simocephalus*, whose populations grew worse when *Daphnia* was the early colonizer and grew best (although still below *Daphnia*) when priority was given to *Simocephalus* (see Figures 1 and 3). This demonstrates priority effects, with the competitive pressure on *Simocephalus* being higher when its competitor arrived earlier in the communities. This superior competitor ability of *Daphnia* could be probably due to the successful establishment of its population through a rapid monopolization of resources (Alonso, 1996; Louette & De Meester, 2007), ability to explore low levels of food (Gliwicz, 1990; Kreutzer & Lampert, 1999; De Meester et al., 2007), and superior filtration rate relatively to *Simocephalus* (Balayla & Moss, 2004; Louette & De Meester, 2007). Despite this superior capacity of *Daphnia*, competitive exclusion (Hutchinson, 1961; Bengtsson, 1986) of *Simocephalus* was not observed here in any scenario. However, the experiment was of relatively short duration and food levels were not very limiting (see Antunes et al., 2003). Also, competition is not such a radical force as e.g. predation (see Louette & De Meester, 2007), which implies that active removal of individuals from populations occurs.

While both populations' growth rate decreased with increasing NaCl concentration, *D. galeata* growth was much more affected at 1.5 g L^{-1}, and this alleviated *S. vetulus* from the pressure of a superior competitor. Consequently, priority effects were nullified, and *Simocephalus* experimental populations grew equally well in all species inoculation order scenarios at 1.5 g L^{-1}. The superior competitive ability of *Simocephalus* at 1.5 g L^{-1} resulted from its higher halotolerance relatively to *Daphnia*. Although previous laboratorial tests showed that the two taxa have similar acute tolerance to salinity (standard acute EC_{50} values (and respective 95% confidence intervals) were 2.81 (2.65-2.99) g L^{-1} for *S. vetulus* and 2.88 (2.73-3.05) g L^{-1} for *D. galeata*), the reproductive EC_{50} (and respective 95% confidence intervals) (unpublished data) for the *S. vetulus* clone used in these experiments was slightly higher (1.28 (1.22-1.33) g L^{-1}) than for the *D. galeata* clone (0.71 (0.64-0.77) g L^{-1}). Thus, our results do not support the hypothesis that priority effects confer some protection to the less tolerant species (in this case, *Daphnia*), similarly to observations in a study with *Microcystis* populations in the presence of grazers (Van Gremberghe et al., 2009). Indeed, in our study, the shift from a *Daphnia*-dominated (0.0 and 0.75 g L^{-1}) to a *Simocephalus*-dominated assemblage (1.5 g L^{-1}) was abrupt. This was mediated by NaCl concentration, which depressed *Daphnia* growth and alleviated the competition over *Simocephalus* (as seen by the lack of an effect of the inoculation order, unlike in ≤ 0.75 g L^{-1} scenarios).

These evidences support the theory that the competition between species can be alleviated under environmental stress (Wilson & Keddy, 1986), favouring the inferior competitor or species that arrive later to the community (Cáceres, 1998; Louette & De Meester, 2007; Van Gremberghe et al., 2009). Consequently, as competitive strength is reduced, decreased impact of priority effects occurs in the presence of a stressor, such as predation (Louette & De Meester, 2007) or limiting food resources (Cáceres, 1998). Similarly to our study, Louette & De Meester (2007) showed that predation was responsible for an inversion of the dominant taxon in experimental communities. Although not as radical as predation (which lead to extinction of some species and hence negative growth rates; Louette & De Meester, 2007), low salinity levels (1.5 g L^{-1}) inverted the competitive outcome in the *Daphnia-Simocephalus* experimental system. The salinity levels at which this occurred are in line with the predictions of Schallenberg et al. (2003), as well as with the NaCl concentrations that elicit reproductive impairment in *Daphnia* (Gonçalves et al., 2007; Loureiro et al., in press).

This study indicates that the higher halotolerance of *S. vetulus* could contribute to its success in disturbed communities, being important in the dynamics of species succession in a progressive scenario of freshwater salinization. In fact, we did find an active population of *S. vetulus* in a brackish lake, as well as evidences of a dormant one in a nearby lake (Loureiro et al., 2012). On the contrary, we could not find populations from the *D. longispina* group (which includes *D. galeata*) in brackish environments. However, care should be taken when generalizing the evidences, because the experimental design was quite simple, including just one competitive pair (*Daphnia-Simocephalus*), forcing the taxa to coexist, despite their distinct ecological niche in nature (Alonso, 1996) and not taking into consideration the genetic variability of the species (one clone per species) (Boersma et al., 1999; De Meester et al., 2007).

In brackish lakes, large herbivores (especially *Daphnia* spp.) tend to be eliminated (Schallenberg et al., 2003); consequently, smaller or more tolerant species dominate (Cáceres, 1998; Emery et al., 2001) but their filtration efficiency is inferior, leading to lake regime shifts from clear to turbid water (Jeppesen et al., 2007). Although *Simocephalus* is a large cladoceran, it is usually restricted to littoral environments and has a sessile behavior

(Alonso, 1996). Consequently, its filtration rate at whole-lake scale may not be efficient in controlling phytoplankton growth (Balayla & Moss, 2004; Louette & De Meester, 2007). Large cladoceran species, and particularly *Daphnia*, play a key role (Jeppesen et al., 2007; Tautz, 2011) in the regulation of primary production in freshwater ecosystems (PEG model; Sommer et al., 1986), because of their efficient algal filtration (Gliwicz, 1990; Kreutzer & Lampert, 1999; Tautz, 2011). So, if the competitive ability of *Daphnia* species is compromised by external factors, such as salinity, the dynamic of species succession could be modified, as shown here, and the ecosystem services provided by these grazers (regulation of biogenic turbidity and prevention of cyanobacterial blooms, as well as nutrient cycling) would be nullified. This is a case where a single species (keystone species) can dramatically alter an ecological process (Covich et al., 1999), in this case grazing rate.

We must recognize that, in a scenario of moderate to intense salinisation, the levels of salinity used in this study are not ecologically relevant. However, saline intrusion may elicit a progressive scenario, particularly in coastal lakes (Schallenberg et al., 2003; Nielsen et al., 2008; Loureiro et al., 2012). In these systems, small increases in salinity may occur due to intermittent inputs of seawater (Hall & Burns, 2002; Schallenberg et al., 2003; Loureiro et al., 2012), but also via saline intrusion in groundwater, as the result of the conjugation of extended droughts (IPCC, 2008; Nielsen & Brock, 2009) and overexploitation of aquifers (Chen et al., 2004; IPCC, 2008). Thus, subtle or progressive changes in salinity may occur in freshwater coastal systems; this clearly justifies the need to assess the ecological consequences of such subtle changes in the resident assemblages, namely zooplankton, whose community structure is predicted to be highly sensitive to saline intrusion (see Hall & Burns, 2002; Schallenberg et al., 2003; Santangelo et al., 2008; Brucet et al., 2010; Duchet et al., 2010). Our results show that altered zooplankton communities will emerge at low salinities, via interference with species sorting and priority effects. Other studies have also shown that the structure of communities reflects the environmental conditions in the moment of species sorting (Cáceres, 1998; Mergeay et al., 2011). The capacity of zooplankton communities – that emerged under saline stress – to cope with other (present or future) stressors could be affected, thus potentially compromising water quality (transparency, cyanobacterial blooms) and ecosystem functioning (e.g. primary productivity, nutrient cycling). Indeed, Wittebolle et al. (2009)

have shown that the initial assemblage structure is a key factor in preserving the resistance to environmental stress and functional stability of an ecosystem.

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Capítulo V

Efeitos de alterações ambientais em populações de invertebrados: uma abordagem prática

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O presente capítulo constitui um manuscrito em publicação em revista nacional com arbitragem científica "Captar".

Efeitos de alterações ambientais em populações de invertebrados: uma abordagem prática

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RESUMO

Mudanças climáticas podem alterar a dinâmica de ecossistemas e, sendo um problema que atualmente motiva preocupações, tem integrado agendas internacionais, designadamente no âmbito das Nações Unidas e da União Europeia. Numa perspetiva de Educação para Desenvolvimento Sustentável (EDS), importa promover o desenvolvimento de competências necessárias à compreensão de dimensões sociais e económicas de mudanças ambientais globais, em particular as relacionadas com comportamentos quotidianos necessários para implementar estratégias de resolução ou mitigação de tais problemas. Neste texto apresentam-se e discutem-se atividades realizadas com alunos do Ensino Secundário, no âmbito do programa de Ocupação Científica de Jovens nas Férias 2010, em que se procurou envolvê-los na aprendizagem de conceitos estruturantes em Biologia, abordando problemas globais atuais, associados a alterações climáticas (*e.g.* salinização de lagoas costeiras de água doce e aumento de temperatura). As atividades práticas realizadas incluíram um *design* experimental: duas espécies de cladóceros de água doce (*Daphnia longispina* e *Daphnia magna*) foram sujeitas a agentes de *stress* abióticos – salinidade e temperatura. Verificou-se que a salinidade, em conjunto com a temperatura, pode causar perturbações em populações das espécies de cladóceros estudadas e, consequentemente, alterar a sua função na regulação das teias tróficas, *e.g.* num lago, comprometendo todo o ecossistema. Com base na observação dos desempenhos dos alunos e na análise das suas respostas a um questionário de avaliação, verificou-se que as atividades práticas realizadas fomentaram o desenvolvimento de competências necessárias para tratar e interpretar dados, assim como para estabelecer relações entre os resultados obtidos e problemas ambientais.

PALAVRAS-CHAVE

Alterações climáticas; salinidade; temperatura; *Daphnia longispina*; *Daphnia magna*; educação científica.

INTRODUÇÃO

Problemas globais atuais, nomeadamente as alterações climáticas relacionadas com o aumento global da temperatura (aquecimento global), têm causado preocupação e discussão a diversos níveis - ambiental, económico e social (UNDP, 2007; UNEP, 2007; AEA, 2010). Em particular, a preocupação ambiental no domínio da água e da biodiversidade tem sido amplamente discutida e analisada por diversas organizações, como a Agência Europeia do Ambiente (AEA) e o Painel Intergovernamental para as Alterações Climáticas (IPCC), resultando em propostas para resolver ou mitigar os problemas que lhes estão associados (IPCC, 2008; AEA, 2010). Numerosos problemas ambientais e económicos, fortemente interrelacionados com perdas de biodiversidade, resultam dos danos causados à biodiversidade e aos ecossistemas e repercutem-se em problemas sociais. Uma vez que travar a perda de biodiversidade requer políticas articuladas e sustentáveis de conservação (UNEP, 2007, 2012), são muito pertinentes as preocupações ambientais ao nível dos ecossistemas aquáticos, que decorrem da previsível subida do nível médio da água do mar provocada pelo aumento da temperatura média do planeta (IPCC, 2008). Adicionalmente, períodos de seca prolongada e a sobre-exploração de aquíferos podem causar redução do nível freático (água doce subterrânea), levando à entrada de água salgada nos aquíferos costeiros (IPCC, 2002, 2008). Consequentemente podem ocorrer intrusões salinas em lagoas costeiras de água doce, provocando o aumento dos valores da salinidade das suas águas (Schallenberg et al., 2003), de formas brusca ou progressiva. Os organismos que vivem nestes sistemas de água doce terão de desenvolver estratégias para fazer face a alterações, esporádicas ou regulares, da sua salinidade. Alguns estudos sugerem que pequenos incrementos de salinidade podem causar alterações profundas na estrutura das comunidades lacustres (Schallenberg et al., 2003; Jeppesen et al., 2007), podendo traduzir-se em perda da biodiversidade local (IPCC, 2002; AEA, 2007, 2010) e, consequentemente levar à alteração do estado trófico destes ecossistemas aquáticos, nomeadamente ao nível da transparência da água (Jeppesen et al., 2007), uma vez que se modificam condições ambientais que regulam o funcionamento das teias tróficas destes ecossistemas.

Numa perspetiva ecológica, o zooplâncton (*e.g.* pulgas de água, *Daphnia* spp.) é um grupo chave no estudo de sistemas aquáticos (Benzie, 2005) e um excelente modelo animal para investigação e ensino em temáticas ambientais, por exemplo relacionadas com alterações climáticas. As pulgas-de-água (nome genérico dado aos animais pertencentes à ordem Cladocera, ver outros artigos da CAPTar – Castro et al., 2009; Claro et al., 2010) são seres consumidores característicos de ecossistemas de água doce (Schallenberg et al., 2003; Gonçalves et al., 2007), assumindo um papel fundamental como reguladores de produção primária, reciclagem de nutrientes e transferência de energia para níveis tróficos superiores (Hall & Burns, 2002; Sarma et al., 2006; Gonçalves et al., 2007; Castro et al., 2009; Claro et al., 2010). Assim, estes organismos regulam o funcionamento das teias tróficas nos ecossistemas de água doce (Jeppesen et al., 1999; Castro, 2007) e são importantes na avaliação da qualidade ambiental desses sistemas (Castro, 2007). O seu desempenho ecológico constitui um indicador importante na monitorização da qualidade ambiental, podendo ser um fator decisivo na tomada de decisões relativas à utilização de recursos pelas populações humanas.

Os dafniídeos (sobretudo *Daphnia*) têm sido utilizados como modelos biológicos em estudos de Ecologia e Ecotoxicologia e apresentam grande potencialidade para se integrarem em recursos educativos. Apesar de serem característicos de água doce, várias populações já foram encontradas em ecossistemas aquáticos de água salobra (salinidade entre 0,1 e 2,3), com alguma ligação com o mar (Jeppesen et al., 1994; Schallenberg et al., 2003; Marques et al., 2006; Santangelo et al., 2008; Loureiro et al., 2012). A ocorrência destes zooplanctontes em água salobra pode estar associada à sua capacidade de adaptação a níveis de salinidade baixos, que requer alterações fisiológicas de osmorregulação, dependentes de fatores genéticos (Arnér & Koivisto, 1993; Aladin & Potts, 1995; Martínez-Jerónimo & Martínez-Jerónimo, 2007). A sua tolerância à salinidade pode também estar associada a fatores ambientais, como a temperatura (Hall & Burns, 2002). A interdependência entre salinidade e temperatura pode ser testada experimentalmente em modelos biológicos, como *Daphnia*, para demonstrar efeitos complexos das alterações climáticas. Estudos desta natureza podem corroborar as evidências apresentadas em estudos paleontológicos que correlacionam períodos mais quentes com níveis mais reduzidos de biodiversidade (UNEP, 2008).

Numa perspetiva socioeconómica, cidadãos, enquanto consumidores, devem desenvolver consciência ambiental e ética sobre problemas ambientais. Na estratégia da CEE/ONU reconhece-se *Educação para Desenvolvimento Sustentável* (EDS) como "*um pré-requisito para se atingir o desenvolvimento sustentável e um instrumento essencial à boa governação, às tomadas de decisão informadas e à promoção da democracia*" (UN, 2005). Reafirmando-se a educação como um direito humano fundamental, reconhece-se que a EDS engloba questões de natureza ambiental, social e económica, pelo que *Educação Ambiental* (EA) deve articular-se e complementar-se "*com outras áreas educativas, numa abordagem integrada conducente à EDS*" (UN, 2005). Como a vida quotidiana dos cidadãos depende da biodiversidade, a sua contribuição para a degradação dos ecossistemas e perda de biodiversidade (muitas vezes irreversíveis) põe em causa o desenvolvimento futuro (UNEP, 2007, 2012).

Assim, alterações climáticas e outras problemáticas, de importância crescente, devem articular-se com ideias estruturantes identificáveis em documentos oficiais para todos os níveis de ensino, procurando que os membros das comunidades escolares desenvolvam competências necessárias à implementação de estratégias de resolução ou mitigação de problemas ambientais (OECD, 2009). Para tal, é necessário promover e desenvolver uma educação básica forte que contemple esses problemas (UNEP, 2007) e valorize as dimensões de educação *pelas* e *sobre* ciências, equilibradamente com educação *em* ciências – educação científica tripolar (Pedrosa & Moreno, 2007). É importante que professores e alunos desenvolvam competências necessárias para decidirem e atuarem mais conscienciosa e fundamentadamente no seu quotidiano, integrando princípios de sustentabilidade e valorizando o desenvolvimento de competências, designadamente de literacia científica (OECD, 2009), como se preconiza em EDS (UN, 2005). Requerendo inovações profundas, integrar EDS em educação científica formal depende prioritariamente da clarificação e articulação de ideias, normalmente não contempladas em perspetivas educativas tradicionais (UNEP, 2007). Tal como sugerem o *Currículo Nacional do Ensino Básico* (CNEB), as *Orientações Curriculares de Ciências Físicas e Naturais* (OCCFN) e os programas de disciplinas de ciências do Ensino Secundário, deve estimular-se o desenvolvimento de competências, nomeadamente competências científicas (Mintzes et al., 2000; OECD, 2009), numa perspetiva complexa que radique na inclusão de princípios de sustentabilidade, na valorização de literacia científica e no desenvolvimento de valores em educação científica para todos (Loureiro et al., 2008; Aikenhead, 2009; Vieira et al., 2011).

O estudo aqui apresentado desenvolveu-se no âmbito de atividades com alunos do Ensino Secundário que se inscreveram no programa de *Ocupação Científica de Jovens nas Férias 2010* (financiado pela Ciência Viva – Agência Nacional para a Cultura Científica de Tecnológica) no Departamento de Biologia e Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro. Os alunos, apoiados por investigadores, tiveram oportunidade de se envolver em atividades práticas relativas à influência da temperatura na tolerância à salinidade em organismos-modelo, representativos dos ecossistemas de água doce, através de ensaios rápidos de tolerância que permitem medir o tempo até à imobilização dos organismos. Utilizaram-se duas espécies de dafniídeos (*Daphnia longispina* e *Daphnia magna)* como modelos biológicos para ajudar a articular problemas globais (*e.g.*, alterações climáticas) e ideias estruturantes em Biologia (*e.g.*, a dinâmica dos ecossistemas). Esta articulação deve constituir uma preocupação das comunidades científicas responsáveis pela construção de conhecimento científico (Loureiro et al., 2008) e requer cooperação entre Universidades e escolas - essencial numa perspetiva de EDS -, de que este estudo é um exemplo.

Assim, recursos didáticos que articulem ideias pertinentes e resultantes da exploração de documentos produzidos por comunidades científicas e/ou de relatórios de organizações internacionais credíveis, podem contribuir para ajudar professores de ciências a clarificar e relacionar conceitos necessários para compreenderem problemas que importam aos cidadãos e, se possível, contribuírem para os resolver ou mitigar - fundamental numa perspetiva de EDS. A construção e utilização de recursos, como o da Figura 1, é importante para promover aprendizagens significativas (Mintzes et al., 2000; Novak, 2000), integrar ideias estruturantes em Biologia e em EDS, e indicar algumas organizações internacionais importantes neste âmbito e fontes credíveis de informação pertinente.

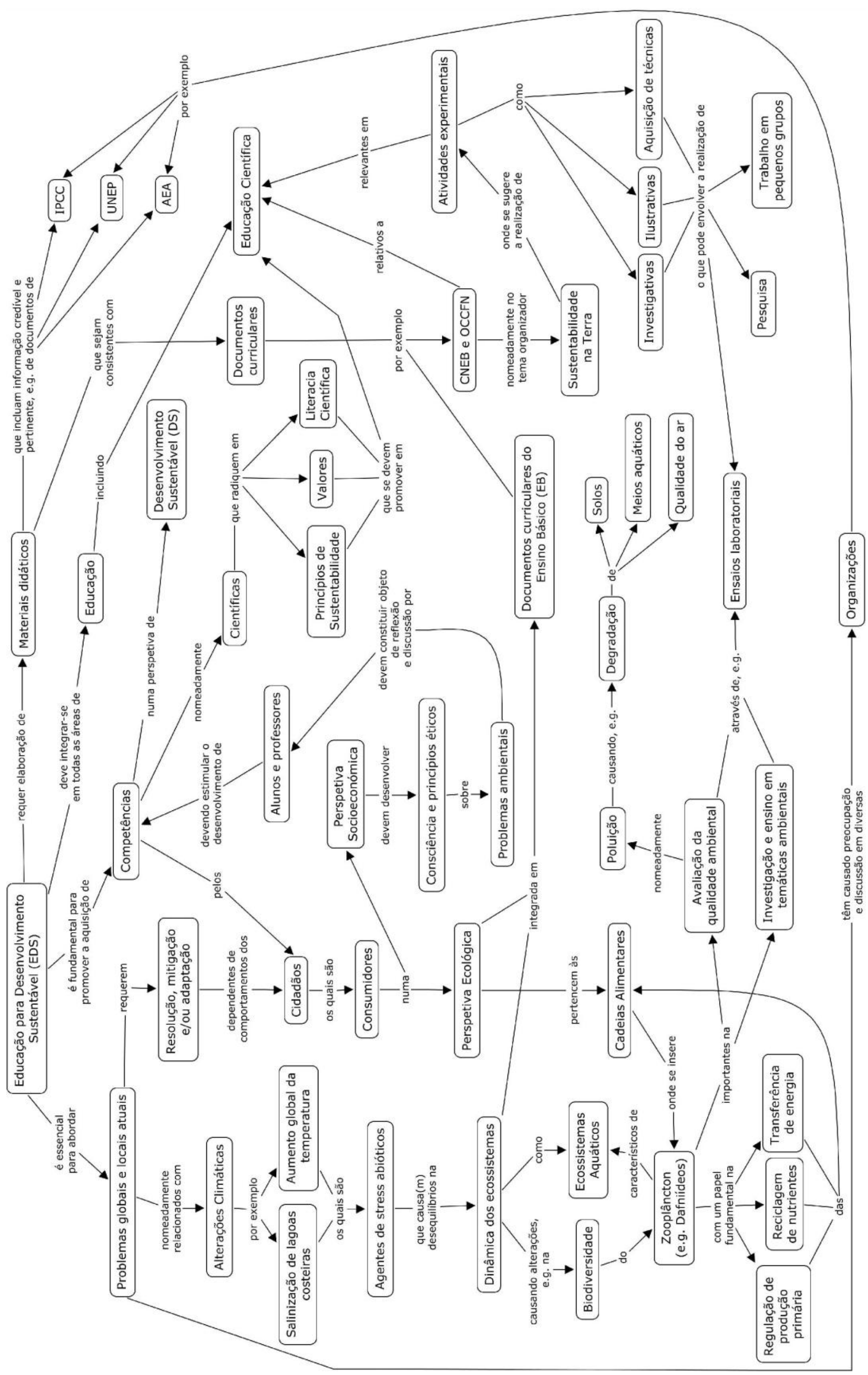

Figura 1 – Diagrama com conceitos inerentes à problemática da biodiversidade, nomeadamente em ecossistemas aquáticos e respetiva regulação, e comportamentos dos cidadãos, visando o seu envolvimento em alternativas mais sustentáveis – centrais em educação para desenvolvimento sustentável.

MATERIAL E MÉTODOS

Descrição das atividades

As atividades do *Programa de Ocupação Científica no Verão* decorreram durante uma semana no Departamento de Biologia da Universidade de Aveiro, conforme previamente planificadas. Nelas participaram quatro alunas de quatro escolas de Ensino Secundário dos distritos de Aveiro e de Coimbra. As alunas tinham idades compreendidas entre 15 e 17 anos e frequentavam o 10º e 11º ano de escolaridade. No primeiro dia, após a receção às alunas, foi feita uma visita ao laboratório onde se iriam realizar as atividades, com apresentação das instalações e familiarização com o material a utilizar. Seguiu-se a apresentação das normas de segurança, uma breve preleção introdutória acerca de conceitos teóricos subjacentes às propostas de atividades e a preparação de meios de cultura a utilizar posteriormente. Nos dias seguintes, foram realizados ensaios experimentais (ver abaixo), registo, interpretação e tratamento de dados, e discussão dos resultados obtidos. Seguidamente foi feita uma reflexão sobre o modo de analisar e de apresentar os dados, com ênfase em construção de gráficos, e discutiu-se meios para o fazer. Após discussão dos resultados obtidos, as alunas escreveram individualmente um relatório sucinto (resultados esperados, metodologias e resultados observados). No final, as alunas avaliaram as atividades realizadas, respondendo individualmente a um questionário escrito correspondente à estrutura da tabela 1 (em que se apresenta uma síntese das respostas) e às frases apresentadas na primeira coluna.

Material biológico

As culturas de *D. longispina* (O.F. Müller) (espécie existente em Portugal) e *D. magna* Straus (espécie padrão, não nativa) foram implementadas a partir de exemplares de campo. Ambas são cultivadas há vários anos no Laboratório de Ecologia de Águas Doces, Ecotoxicologia e Risco (LEADER, http://leader.web.ua.pt), no Departamento de Biologia da Universidade de Aveiro (http://www.ua.pt/bio). As culturas de *D. longispina* foram implementadas pela equipa do LEADER (clone EV20, ver Antunes et al., 2003), ao passo que *D. magna* foi obtida a partir de culturas laboratoriais padrão em laboratórios europeus.

Ambas as espécies foram mantidas em laboratório ao longo de várias gerações em condições de cultura controladas, com temperatura de 20±2ºC e fotoperíodo de 16h$^{\text{LUZ}}$: 8h$^{\text{ESCURO}}$. Os organismos foram cultivados em frascos de vidro de 1 L, em grupos de aproximadamente 40 indivíduos, usando como meio de cultura água reconstituída moderadamente rica em cálcio e magnésio ($NaHCO_3$: 81 mg L^{-1}, $CaSO_4.2H_2O$: 60 mg L^{-1}, $MgSO_4$: 60 mg L^{-1} e KCl: 4 mg L^{-1}) (ASTM, 1980; USEPA, 2002). De forma a proporcionar alguns oligoelementos essenciais, o meio de cultura foi aditivado com um extrato orgânico padronizado (Baird et al., 1989). Instituições internacionais (OCDE e USEPA) recomendam este meio semiartificial (água reconstituída e extrato) para o cultivo de dafniídeos.

A renovação do meio de cultura foi efetuada de 2 em 2 dias. Com a mesma periodicidade, os organismos foram alimentados com a microalga *Pseudokirchneriella subcapitata*, em concentrações de 1,5 x 10^5 e 3,0 x 10^5 célula mL^{-1}, para *D. longispina* e *D. magna*, respetivamente. As culturas foram renovadas ciclicamente com juvenis nascidos entre a 3ª e a 5ª ninhada. O cultivo de *Daphnia* assenta na sua capacidade de reprodução assexuada, que garante a produção contínua de descendência geneticamente idêntica. Este facto, aliado à padronização das condições de cultura, minimiza a variabilidade das respostas dos organismos entre ensaios experimentais.

Ensaios de sensibilidade a agentes de *stress*

Nos ensaios de sensibilidade, monitorizou-se o tempo à imobilização de *D. longispina* e *D. magna* durante a exposição ao *stress*. Os organismos utilizados tinham 4 a 5 dias e foram recolhidos entre a 3ª e a 5ª ninhada das respetivas culturas. Normalmente, nos ensaios com dafniídeos utilizam-se juvenis com menos de 24 h (neonatos), por estes representarem o estado mais sensível do seu desenvolvimento. Todavia, a utilização de organismos maiores facilita a sua visualização e manuseamento.

De forma a testar o efeito da temperatura sobre a tolerância à salinidade, submeteram-se os organismos a concentrações fixas de cloreto de sódio (NaCl: 6 g L^{-1} para *D. longispina* e 8 g L^{-1} para *D. magna*), que foram definidas com base no conhecimento prévio da tolerância de ambas as espécies a este agente de *stress* (NaCl). As soluções foram preparadas dissolvendo 6 g e 8 g de NaCl, respetivamente, em 1 L de meio de cultura (ver acima). Foram utilizadas duas condições experimentais: temperatura ótima (20ºC), à qual os organismos estavam já aclimatados, e temperatura elevada (30ºC). Os ensaios decorreram em placas de 24 poços (4 linhas x 6 colunas) – ver Figura 2. Em cada poço colocaram-se 2 mL da respetiva solução de cloreto de sódio (NaCl). Posteriormente, colocou-se um organismo em cada poço até perfazer um total de 12 organismos de cada espécie (as duas primeiras linhas com *D. longispina* e as outras duas com *D. magna*). Cada placa constituiu uma réplica experimental, tendo-se utilizado 3 réplicas por cada condição experimental. Para cada temperatura, realizou-se o respetivo controlo negativo (meio de cultura sem adição de NaCl), de forma a dissociar o efeito isolado da temperatura na imobilização dos organismos. A realização de controlos negativos permitiu também assegurar a qualidade do lote de organismos utilizados; a placa do controlo consistia em 12 organismos de cada espécie individualmente expostos às mesmas condições de temperatura (20º e 30ºC), mas na ausência de NaCl.

Os organismos foram observados à lupa, em intervalos de 30 min, desde o início dos ensaios até à imobilização de todos os indivíduos; em cada observação, os indivíduos foram considerados imóveis quando não respondiam ao estímulo do observador (toque subtil e repetido com agulha de disseção). Para cada indivíduo, foi registado o tempo à imobilização, em minutos (ver mais detalhes em Loureiro et al., 2012). Os controlos foram monitorizados com uma periodicidade mais alargada (em intervalos de 2 h ou de 3 h) apenas para confirmar a validade do ensaio.

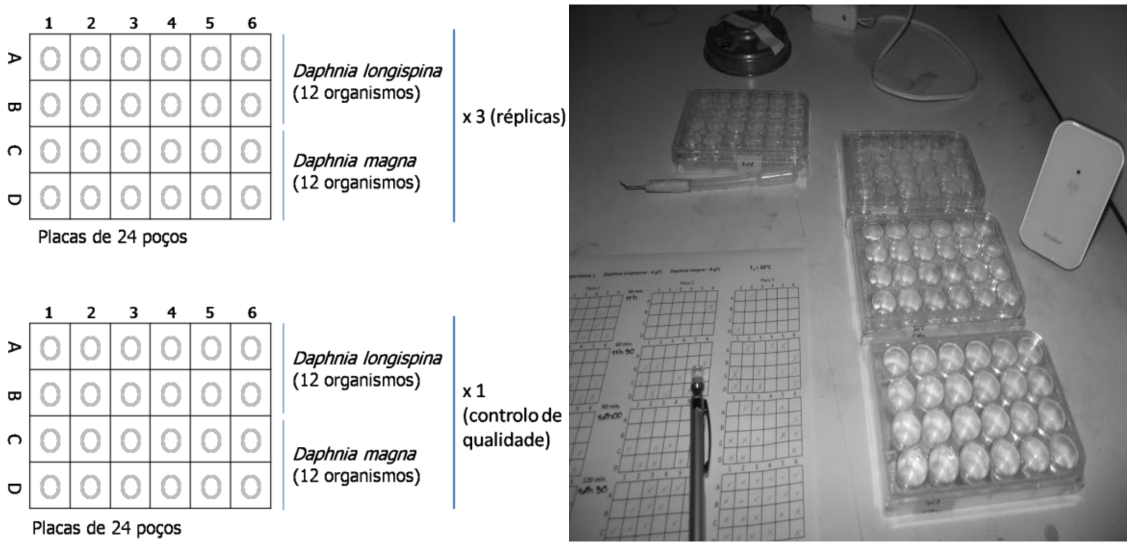

Figura 2 – Esquematização da logística necessária ao desenvolvimento experimental do ensaio.

Análise Estatística

Realizaram-se testes de t (bilateral ou com duas caudas) para observações independentes e grupos com variâncias iguais[1] para determinar a significância estatística das diferenças de sensibilidade observadas – expressas em tempo à imobilização (min) – entre as duas temperaturas testadas. As diferenças de tolerância foram consideradas significativas quando a probabilidade (p) associada ao teste de t foi inferior a 0,05 (i.e., 5%).

RESULTADOS

Desenvolvimento de competências pelo grupo de alunas

As alunas participaram de forma empenhada nas atividades propostas, manifestando interesse pelo trabalho, colocando questões e procurando responder a todas as solicitações dos investigadores. Todas atribuíram um grau de dificuldade adequado ao trabalho realizado e a avaliação global das atividades foi *Excelente* para três alunas e *Boa* para uma aluna. Quanto à duração do estágio, foi considerada *Adequada* por três alunas e *Curta* por uma aluna. O horário foi considerado *Bom* por todas as alunas.

Na avaliação final da semana de trabalho na Universidade, as alunas referiram várias situações de que mais gostaram, *e.g.*: "*trabalhar em laboratório e com equipamentos que ainda não tinha tido hipótese de trabalhar*"; "*ver de perto como se processa uma investigação científica*"; "*trabalhar em laboratório (...) tendo uma certa autonomia ao realizar o procedimento experimental aprendendo assim processos e técnicas que poderão ser necessárias para aplicar em laboratório no próximo ano letivo*"; "*(...) poder manipular instrumentos de observação e investigação a que não tenho acesso na escola*"; "*a dedicação exigida neste estágio foi outro aspeto importante que me tornou, com certeza, mais esforçada e responsável, o que é uma mais-valia para o meu futuro académico e profissional*". Gostaram menos de: "*não ter a oportunidade de continuar a estudar e a fazer experiências para perceber quais as razões que levaram a ter alguns resultados inconclusivos*"; "*da parte teórica em que descrevemos os materiais e métodos utilizados no procedimento experimental*". As maiores dificuldades apontadas foram: "*perceber alguns termos mais técnicos mas, rapidamente essa dificuldade foi superada*

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[1] http://office.microsoft.com/pt-pt/excel-help/acerca-das-ferramentas-de-analise-estatistica-HP005203873.aspx (consultado em Maio de 2011)

com a ajuda dos investigadores”; “*manusear algum equipamento*”; “*trabalhar em folhas de cálculo com fórmulas e também alguma dificuldade em fazer gráficos*”. Sugeriram: “*extensão do tempo de estágios*”, “*continuar a oferecer estágios para o ano para que os alunos tenham oportunidade de aprender mais durante as suas férias de forma divertida*” e “*permitir a participação de maior número de alunos em cada estágio*”.

Tabela 1 – Respostas das alunas do *Programa de Ocupação Científica* a perguntas do questionário de avaliação das atividades realizadas.

Afirmação	**Concordo Totalmente**	**Concordo**	**Discordo**	**Discordo totalmente**
Este estágio ajudou-me a escolher a minha carreira futura		3	1	
Tive oportunidade de participar no trabalho científico desenvolvido por profissionais	2	2		
A componente prática do estágio foi importante: aprendemos a teoria e como aplicá-la na prática	2	2		
Os investigadores incentivaram-nos a refletir e a questionarmo-nos sobre todo o processo	4			
Este estágio permitiu-nos executar as técnicas estudadas em vez de ficarmos apenas a observar “como se faziam as coisas”	4			
Formou-se um espírito de equipa fantástico que ajudou ao desenvolvimento do trabalho	4			
Adquiri conhecimentos que podem vir a ser bastante importantes para o meu futuro profissional	3	1		
Os investigadores estiveram sempre disponíveis para nos ajudar e para esclarecer todas as dúvidas que iam surgindo	4			
Os investigadores ajudaram-nos a perceber para que serve o que aprendemos na teoria	3	1		
Trabalhei com materiais e equipamentos que não existem na minha escola	1	3		
Este estágio permitiu-me “entrar na pele” de um cientista, vivendo “in loco” o dia-a-dia da atividade científica	4			
Gostaria de recomendar este estágio aos meus amigos	4			

A tabela 1 mostra que as alunas consideraram importante o contato com investigadores da Universidade e a oportunidade de aprenderem novas técnicas experimentais autonomamente, pois, de uma forma geral, as apreciações relativamente às atividades foram bastante positivas. Em alguns pontos há unanimidade nas respostas, ressaltando-se a concordância com o papel dos investigadores no incentivo à reflexão e à interrogação, relativamente a terem executado as técnicas necessárias ao desenvolvimento da atividade experimental e relativamente ao trabalho de equipa com os investigadores, o que permitiu perceber o trabalho diário cooperativo de uma equipa de investigação.

Ensaios de sensibilidade a agentes de *stress*

Perante concentrações relativamente baixas de NaCl (*e.g.* na água do mar, a concentração de sais – sobretudo NaCl – é superior a 30 g L^{-1}), os dafniídeos foram muito sensíveis, tendo morrido em minutos ou horas. Os resultados dos tempos à imobilização não podem ser comparados entre espécies, na medida em que se usaram concentrações de NaCl diferentes para cada espécie, face às suas diferentes tolerâncias. Contudo, é possível comparar o perfil de sensibilidade ao NaCl das espécies quando expostas às diferentes temperaturas (Figura 3). Nesse aspeto, os resultados foram contraditórios, com *D. longispina* ligeiramente mais sensível ao NaCl (*i.e.*, demorou menos tempo a imobilizar-se) a 30ºC, enquanto *D. magna* foi significativamente mais sensível ao NaCl a 20ºC. Estatisticamente, esta observação foi marginalmente não significativa para *D. longispina* (teste *t*: $p = 0,07$) e significativa ($p = 0,01$), para *D. magna*.

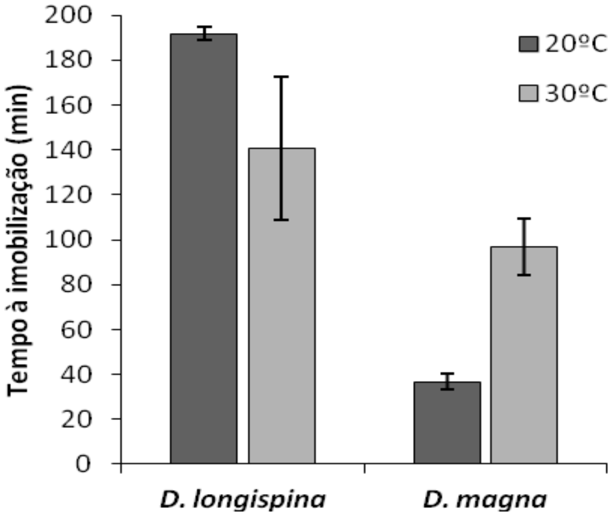

Figura 3 – Tempo à imobilização em *Daphnia* spp. expostas a uma concentração letal de NaCl (6 g L^{-1} para *D. longispina* e 8 g L^{-1} para *D. magna*) a 20 ºC e a 30 ºC.

DISCUSSÃO

Análise do desenvolvimento de competências pelo grupo de alunas

Numa perspetiva de EDS e de educação científica tripolar (Pedrosa & Moreno, 2007), relacionaram-se ideias estruturantes em Biologia com problemas globais, visando contribuir para estimular o desenvolvimento de competências necessárias para utilizar conhecimento científico e tecnológico em resolução de problemas. As atividades realizadas integraram uma abordagem experimental, envolveu cooperação entre investigadores de uma Universidade e quatro alunas do ensino secundário que participaram no estágio e revelou-se importante para a sua motivação e, consequentemente, para aprenderem, como evidenciam as respostas acerca do manuseamento dos materiais e a autonomia e entusiasmo revelados na execução das tarefas.

As maiores dificuldades reveladas pelas alunas resultaram de falta de competências específicas em ciências, como para identificar e compreender questões científicas, utilizar meios para analisar dados, explicar cientificamente fenómenos e formular conclusões com base em evidências (OECD, 2009). O desenvolvimento de competências científicas que habilite os alunos para tomar decisões conscientes e fundamentadas, numa perspetiva de Educação para Desenvolvimento Sustentável, é fundamental para promover literacia científica e deve ocorrer ao longo de toda a escolaridade (UN, 2005). O reconhecimento das situações que envolvem ciências e tecnologias, a compreensão do mundo natural com base em conhecimentos científicos e as atitudes perante as ciências (interesse e motivação por questões científicas) são outras dimensões importantes de literacia científica (OECD, 2009). Os recursos utilizados pelos professores serão relevantes para eles próprios desenvolverem competências, incluindo científicas, que os habilitem a estimular os alunos em processos idênticos (McNeill & Krajcik, 2008). Um professor que não se limite a transmitir conteúdos e utilize estratégias diferenciadas que interliguem ciências e tecnologias com a vida quotidiana, melhora a literacia científica dos seus alunos (UN, 2005; OECD, 2009) e pode estimulá-los a participar ativa e fundamentadamente na sociedade (Galvão & Freire, 2004; UN, 2005). Assim, sustentadas por conhecimentos científico-tecnológicos sólidos, os seus contributos para resolver ou mitigar problemas atuais, particularmente locais com implicações globais, serão mais eficazes e terão impactos mais fortes.

Numa perspetiva de EDS, as comunidades científicas têm um papel fundamental no desenvolvimento de abordagens de questões local e globalmente relevantes, importantes para vencer barreiras de organização disciplinar tradicional (Loureiro et al., 2008). O desenvolvimento de parcerias entre universidades e escolas, além de ajudar a superar dificuldades, como as apresentadas pelas alunas, é essencial para abordar questões local e globalmente relevantes e construir conhecimento nesse âmbito. Além disso, seria importante que no ensino superior existissem disciplinas de ciências de largo espetro, tendo como eixo estruturante a orientação CTS – Ciência-Tecnologia-Sociedade (Martins, 2002) e se implementassem reformas educativas que promovessem o estabelecimento de parcerias entre investigadores e professores dos ensinos básico e secundário. Assim, em ensino das ciências poderia utilizar-se formas mais aliciantes de abordar questões atuais, aproximando os jovens das ciências e tecnologias e contribuindo para melhorar a qualidade de vida, principalmente de grupos mais desfavorecidos (Martins, 2002; Vieira et al., 2011).

O desenvolvimento de trabalho prático pode também motivar os alunos, quando continuamente estimulados, uma vez que, de contrário, podem desinteressar-se por aprender ciências (Abrahams, 2009; OECD, 2009). A realização de atividades em pequenos grupos é também importante para motivar os alunos e melhorar as suas aprendizagens, pois permite desenvolver capacidades de argumentação fundamentada, por exemplo, em contextos de aulas de ciências (Bennett et al., 2010). É, pois, imperioso desenvolver, testar e avaliar recursos educativos inovadores que, numa perspetiva de EDS e articulando conteúdos disciplinares, enfatizem sustentabilidade ambiental e valorizem as dimensões de educação *pelas* e *sobre* ciências, equilibradamente com a de educação *em* ciências. Recursos educativos inovadores deveriam remeter para padrões e qualidade de vida dos cidadãos, nomeadamente de alunos a que se destinam, e suas relações com problemas ambientais, perda de biodiversidade e imperativos de promoção de qualidade ambiental daí decorrentes. No entanto, existem constrangimentos na implementação de estratégias que recorram a recursos inovadores (Martins, 2002), tal como se constatou nas dificuldades referidas pelas alunas, por exemplo de construção de folhas de cálculo e de gráficos, assim como de análise e discussão de resultados (ver acima).

Ensaios de sensibilidade a agentes de *stress*

Vários estudos (*e.g.* Teschner, 1995; Sarma et al., 2006; Martínez-Jerónimo & Martínez-Jerónimo, 2007; Santangelo et al., 2008) mostraram que a halotolerância varia de espécie para espécie. A temperatura também influencia o funcionamento fisiológico dos organismos e das teias tróficas em que estes se inserem (Van Doorslaer et al., 2007; Van Doorslaer et al., 2010). A interação entre os dois agentes de *stress* abióticos, salinidade e temperatura, mostrou interferir com as respostas dos organismos testados, no entanto os resultados não foram consistentes entre espécies. No caso de *D. longispina*, a sua sensibilidade parece não ter sido afetada pelo aumento de temperatura, mas sugere um aumento de sensibilidade a temperatura superior; pelo contrário, no caso de *D. magna*, há um claro efeito protetor da temperatura. Os resultados referentes a *D. longispina* confirmam o observado por Hall e Burns (2002), enquanto os relativos a *D. magna* contradizem a tendência de aumento de sensibilidade à salinidade com o incremento de temperatura. Estas evidências permitiram ajudar as alunas a aperceberem-se de que as simulações experimentais estão sujeitas a erros e contradições, especialmente quando utilizam organismos vivos.

Estas experiências terão que ser repetidas mais vezes de modo a verificar se o padrão obtido é consistente; só então, será possível diminuir a incerteza associada a resultados contraditórios. Não obstante, as diferentes espécies possuem mecanismos fisiológicos diferentes em termos de termorregulação (Van Doorslaer et al., 2007; Van Doorslaer et al., 2010) e osmorregulação (Aladin, 1991; Arnér & Koivisto, 1993; Aladin & Potts, 1995), pelo que é possível que espécies aparentadas mas diferentes (como *Daphnia* spp.) respondam diferentemente aos mesmos estímulos ambientais.

Estes resultados mostram que num ecossistema de água doce que esteja sujeito a flutuações ambientais, como incrementos de salinidade e de temperatura num lago, haverá perturbações na regulação da teia trófica do lago, a qual depende da tolerância dos organismos a essas flutuações. Dado que os organismos testados, devido ao seu papel central nas cadeias alimentares, desempenham funções de regulação entre os diferentes níveis tróficos, pode ocorrer o colapso ecológico do sistema na sua ausência (Jeppesen et al., 1994; Lampert, 2006; Sarma et al., 2006; Jeppesen et al., 2007), comprometendo o funcionamento do ecossistema e os serviços que ele proporciona aos humanos.

Aplicações e perspetivas futuras

O estudo da dinâmica de ecossistemas e de perturbações destes, como variações de temperatura e de salinidade, são assuntos contemplados no *Currículo Nacional do Ensino Básico*, enquadrados no tema organizador “Sustentabilidade na Terra” (DEB, 2001a). Deste modo, as atividades aqui apresentadas podem ser adaptadas pelos professores nas escolas para serem desenvolvidas, de acordo com o nível do ensino, de modo a promover o desenvolvimento de competências específicas de literacia científica dos alunos, tal como preconizado em documentos oficiais para o ensino das ciências (*e.g.* DEB, 2001a, 2001b). Assim, o envolvimento dos alunos em atividades práticas experimentais pode ser motivador e facilitar a aprendizagem de conceitos e o desenvolvimento de competências, que dependem do problema em estudo e do grau de abertura das atividades. É importante que mais sugestões de atividades práticas, por exemplo decorrentes de trabalhos de investigação em universidades, cheguem ao conhecimento de professores e de alunos, inseridas em manuais escolares ou noutros documentos.

No futuro, será desejável construir e desenvolver este tipo de atividades em parceria com professores dos ensinos básico e secundário, de diferentes áreas disciplinares, de modo a incentivar articulações interdisciplinares. A participação de professores dos ensinos básico e secundário neste tipo de atividades, além de fundamental para a sua validação, proporciona oportunidades de formação e desenvolvimento profissional, numa perspetiva de educação científica tripolar, ou, no caso de envolvimento apenas de atividades de biologia, nas dimensões de educação em biologia, sobre biologia e pela biologia. Em qualquer dos casos, o interesse de professores por atividades do tipo das aqui apresentadas e o seu envolvimento nelas, permite-lhes desenvolver competências naquelas três dimensões, que se afigura essencial para poderem ajudar os seus alunos a envolverem-se em atividades que similarmente lhes permitam aprender desenvolvendo competências numa perspetiva de educação científica tripolar orientada para a integração de EDS em áreas curriculares.

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Considerações finais

Os ecossistemas estão sujeitos às mais variadas flutuações ambientais (Bijlsma & Loeschcke, 2005), nomeadamente como consequência das alterações climáticas que se têm intensificado recentemente (*e.g.* Hall & Burns, 2002; Schallenberg et al., 2003; IPCC, 2008; Van Doorslaer et al., 2010; Chen & Stillman, 2012). Estas flutuações podem induzir mudanças a longo prazo nas populações e, consequentemente, na estrutura e dinâmica funcional dos ecossistemas (Bijlsma & Loeschcke, 2005; Van Doorslaer et al., 2010; Chen & Stillman, 2012). Assim, importa compreender qual o impacto das flutuações ambientais no equilíbrio, no funcionamento e nos serviços dos ecossistemas, uma vez que estes estão dependentes da interação entre os organismos (interações bióticas) e entre estes e os fatores abióticos (Bijlsma & Loeschcke, 2005; Castro, 2007). As alterações ambientais podem ocorrer de forma brusca e esporádica, não dando tempo aos organismos para se adaptarem, sobrevivendo, nestas situações, apenas os organismos mais tolerantes. No entanto, num cenário de *stress* ambiental progressivo e a longo prazo, é importante compreender a capacidade de resposta e resiliência das populações, pois têm implicações na estrutura e dinâmica populacional, assim como no funcionamento dos ecossistemas, como referido acima. Nesse sentido, estudos acerca da dinâmica populacional centrados na análise das consequências das alterações ambientais tornam-se bastante pertinentes para dar respostas a questões relevantes (*e.g.*, funcionamento dos ecossistemas, interações tróficas, qualidade do *habitat*) relacionadas com o equilíbrio dos ecossistemas. A dinâmica das populações está dependente da plasticidade e adaptabilidade dos organismos a alterações ambientais (Bijlsma & Loeschcke, 2005). Por exemplo, as flutuações ambientais causam perturbações na dinâmica sazonal do zooplâncton, o que interfere com os mecanismos de regulação da teia trófica de um lago, podendo afetar a qualidade da água e a sua utilização pelos humanos (Boavida, 2000; Castro, 2007). As respostas das populações ao *stress* ambiental dependem da capacidade de adaptação dos indivíduos a perturbações do *habitat*, recorrendo à plasticidade fenotípica (Hart & Bychek, 2011). Por outro lado, a variabilidade genética intra-populacional também influencia a dinâmica populacional (Boersma et al., 1999; De Meester et al., 2002), o que, em última análise, determina a estrutura populacional em ecossistemas de água doce.

Assim, pelos motivos sumariamente indicados, a presente investigação pretendeu explorar a adaptabilidade e sensibilidade de populações de organismos dulçaquícolas (cladóceros) à mudança ambiental, incluindo agentes de *stress* abióticos (salinidade, temperatura e sua interação) e sua influência nas inter-relações bióticas (competição). Parte da novidade da investigação apresentada neste documento prende-se com as abordagens utilizadas, onde os efeitos dos agentes de *stress* não são analisados isoladamente, mas sim integrados numa perspetiva multifatorial, onde, por exemplo, é considerada a influência da adaptação prévia ao agente de *stress* ou o seu potencial efeito sobre as interações inter-específicas (ver mais adiante). Assim, a presente tese aborda a temática das respostas populacionais a mudanças ambientais numa perspetiva mais realista, mas também mais distante do formato clássico dos estudos ecotoxicológicos – que tradicionalmente abordam os efeitos do *stress* ambiental de forma isolada.

Os cladóceros são constituintes do zooplâncton dulçaquícola, desempenhando um papel central nas teias tróficas enquanto herbívoros, responsáveis pela transferência de energia dos produtores para o topo das teias tróficas aquáticas. Estes organismos constituem bons modelos biológicos na avaliação do estado ecológico dos ecossistemas aquáticos, nomeadamente de água doce (Lampert, 2006). A avaliação da sua capacidade de adaptação a alterações nas condições ambientais foi testada experimentalmente, quer ao nível da variabilidade genotípica das populações, quer ao nível da plasticidade fenotípica dos organismos. Assim, especialmente por existirem poucos trabalhos que estudem a capacidade de adaptação em populações de *Simocephalus* sp., apresentou-se neste trabalho informação importante acerca das respostas destes organismos a agentes de *stress* abióticos (salinidade) e interações bióticas (competição). Esta espécie existe essencialmente em zonas litorais e associada a macrófitas (Alonso, 1996). Embora não habite as zonas pelágicas de lagos, este organismo tem grande importância na regulação das cadeias alimentares de um lago com vegetação, pois é um herbívoro filtrador e, portanto, participa também na regulação da quantidade de alimento disponível num lago, contribuindo para a transparência da água (Jeppesen et al., 1999).

Embora o efeito da salinidade nas populações de cladóceros já tenha sido avaliado em alguns estudos (*e.g.* Schallenberg et al., 2003; Sarma et al., 2006; Martínez-Jerónimo & Martínez-Jerónimo, 2007), o presente trabalho permitiu perceber que, num cenário de intrusão salina progressiva em lagoas costeiras de água doce, existem indivíduos capazes de tolerar meios com alguma salinidade, o que poderá ser importante na avaliação da resiliência dos ecossistemas dulçaquícolas. Paralelamente, foram observadas diferenças, embora pouco acentuadas, ao nível da halotolerância aguda a níveis letais de salinidade entre os genótipos das populações de água doce e de água salobra (ver capítulo I). Os genótipos da população de água salobra apresentaram maior capacidade de sobrevivência a níveis mais elevados de salinidade quando comparados com os genótipos das populações de água doce. Estes resultados derivam apenas da variabilidade genotípica destas populações, pois foram retiradas influências ambientais e maternais, devido à manutenção das populações em laboratório durante diversas gerações. Portanto, encontraram-se evidências de mecanismos de halotolerância geneticamente determinados (De Meester, 1996a, 1996b; Boersma et al., 1999; De Meester et al., 2002), com elevada importância na capacidade de resposta das populações a alterações ambientais, como o aumento de salinidade (Schallenberg et al., 2003).

Perante estes resultados, tornou-se importante estudar a halotolerância dos genótipos mais extremos, em cenários sub-letais (capítulo II). Desta avaliação verificou-se que não parece haver adaptação local da população de água salobra, perante um cenário de intrusão salina gradual e níveis baixos de salinidade. Se os genótipos de água salobra tivessem sofrido adaptação local no seu contexto original, teriam de apresentar maior halotolerância crónica do que os genótipos de água doce. Embora não se tenha comprovado a hipótese de adaptação local (De Meester, 1996a, 1996b; Boersma et al., 1999; Cousyn et al., 2001), confirmou-se que uma salinização gradual e progressiva causa perturbações nos diferentes genótipos testados, assim como influencia o crescimento populacional, interferindo, consequentemente, com as comunidades bióticas em que se inserem. Assim, num cenário de intrusão salina, a estrutura e dinâmica populacional estarão dependentes do *pool* genético e da plasticidade fenotípica (ex.: em termos de osmorregulação) das populações residentes, face ao agente de *stress*. Neste caso, a salinidade parece assumir um papel importante como um fator seletivo e modulador de evolução na estrutura genética e diversidade intra-populacional de cladóceros em ambientes flutuantes (Bijlsma & Loeschcke, 2005).

A confirmação de que a resposta das populações a alterações ambientais também está dependente da sua capacidade de adaptação fenotípica foi confirmada ao avaliar as consequências das condições de cultura sobre dois agentes de *stress* abióticos (capítulo III). Assim, procurou-se perceber quais os efeitos interativos da salinidade e da temperatura em cenários de aclimatação progressiva numa população de *Daphnia galeata*, verificando-se que o aumento de temperatura tende a diminuir a halotolerância dos organismos nas primeiras gerações expostas a estes agentes de *stress*. No entanto, verificou-se que, ao fim de algumas gerações de aclimatação, o cenário se alterou, e as culturas simultaneamente pré-aclimatadas a ambos os agentes de *stress* já não seguiram o mesmo padrão dependente da temperatura. Assim, deve-se ter em conta que as possíveis alterações nas respostas dos organismos dependem da sua capacidade de se adaptarem ao longo das gerações, mas também do grau de variabilidade das alterações ambientais (Chen & Stillman, 2012).

Por outro lado, é importante compreender também os impactos para além da população, ao nível das comunidades; neste sentido, avaliaram-se as respostas de comunidades experimentais de cladóceros sujeitas a diversos níveis de salinidade, deste modo avaliando a interação entre um agente de *stress* abiótico e uma relação inter-específica (competição). No capítulo IV deste trabalho, a avaliação desta interação demonstrou que, a baixos níveis de salinidade, ocorre uma inversão da resposta das populações à interação competitiva, alterando o desfecho competitivo entre *Daphnia galeata* e *Simocephalus vetulus*. Apesar de *Daphnia* sp. se ter revelado um competidor superior em condições não alteradas, níveis baixos de salinidade afetaram o seu crescimento populacional e diminuíram a pressão competitiva sobre a espécie menos competitiva (*S. vetulus*). No entanto, esta inversão dependeu da ordem de chegada das espécies (efeitos prioritários). Assim, subtis alterações ambientais podem causar efeitos indiretos nos mecanismos ecológicos, afetando a composição da comunidade e, ulteriormente, a regulação da produção primária – afetando o funcionamento dos ecossistemas aquáticos e a qualidade da água (Jeppesen et al., 2007).

Numa perspetiva de Educação para Desenvolvimento Sustentável (EDS) (Tilbury, 2011), no que diz respeito a preocupações ambientais, atendendo às previsões de alterações climáticas (IPCC, 2008), o conhecimento científico produzido por comunidades científicas pode (e deve) servir de base ao desenvolvimento de atividades com alunos e professores. Desta forma, é possível estimular o desenvolvimento de competências científicas (OECD, 2009), que radiquem em princípios de sustentabilidade, valores e literacia científica, que se devem promover em educação científica. Portanto, enquanto professora considero relevante e importante que o conhecimento produzido por comunidades científicas seja incorporado em documentos oficiais para o ensino em Portugal. Para isso as comunidades científicas devem estar disponíveis para a produção de materiais didáticos, como mapas de conceitos, que incluam informação credível e pertinente e seja consistente com documentos curriculares. Assim, com o trabalho desenvolvido no capítulo V procurou-se promover o desenvolvimento de competências científicas de alunos, envolvendo-os na aprendizagem de conceitos estruturantes em Biologia e estabelecendo contacto com conhecimento produzido por comunidades científicas. Assim, partindo de conhecimento produzido e apresentado nos capítulos anteriores, utilizaram-se metodologias semelhantes na avaliação da halotolerância aguda de *Daphnia magna* e *Daphnia longispina* sob a influência da temperatura. Desta forma, foi possível sensibilizar as alunas para a importância das atividades de investigação sobre os efeitos de flutuações ambientais na dinâmica das populações. Mais ainda, estas flutuações são causadas por alterações climáticas (como a salinização de ecossistemas de água doce e o aumento de temperatura), nas quais os cidadãos têm tido responsabilidade. Por outro lado, esta abordagem prática assenta em temáticas abordadas no ensino básico, nomeadamente sobre ecossistemas (interação entre os seres vivos e o ambiente, fluxo de energia e ciclo de matéria e perturbações no equilíbrio dos ecossistemas) e gestão sustentável de recursos (DEB, 2001a, 2001b). Consequentemente, este tipo de atividades tornam-se relevantes no sentido de promover e aumentar a motivação dos alunos, desenvolver competências científicas e melhorar as suas aprendizagens (Abrahams, 2009; OECD, 2009; Vieira et al., 2011). De facto, esta atividade permitiu consciencializar as alunas para o erro que envolve as atividades de investigação, reforçando a componente de educação sobre ciências, normalmente descurada no ensino formal, e necessária para uma EDS, de caráter tripolar (em, sobre, pelas ciências). Por outro lado, o desenvolvimento de competências científicas permite tomadas de decisão conscientes e fundamentadas em questões quotidianas de cidadãos, em particular alunos e professores (Aikenhead, 2009; Vieira et al., 2011).

Portanto, o trabalho apresentado nesta tese permite chamar a atenção das comunidades científicas, e dos cidadãos em geral, para a necessidade de estarem atentos aos efeitos de pequenas alterações ambientais, maioritariamente causadas por alterações climáticas. Estas constituem um problema global atual, que tem consequências nefastas ao nível local, nomeadamente no funcionamento dos ecossistemas e nos serviços que estes proporcionam. De facto, também a população humana é afetada por estas modificações, devendo tomar atitudes e decisões conscientes e fundamentadas no seu quotidiano, para resolver ou mitigar estes problemas, dos quais de certa forma são responsáveis. Por outro lado, este trabalho é também relevante para a compreensão das consequências dos efeitos interativos entre diversos fatores nos ecossistemas, tornando-se importante continuar estudos incrementando o realismo ecológico dos mesmos (utilizando comunidades experimentais, mesocosmos). Estes estudos podem ser importantes para, de uma forma parcialmente controlada, compreender a capacidade de resposta e resiliência das populações e comunidades biológicas às flutuações ambientais resultantes de alterações climáticas, englobando as componentes genotípica e fenotípica da sua adaptabilidade e suscetibilidade.

Tendo em conta a importância da aclimatação prévia dos organismos para uma avaliação mais realista dos efeitos de agentes de *stress*, sugere-se que em futuras investigações se estudem as respostas agudas e crónicas de diferentes genótipos de populações de dafniídeos com flutuações ambientais, após adaptação aos agentes de *stress*. O grande desafio seria incorporar no desenho experimental, para além da componente fenotípica, a componente de seleção dos indivíduos (alterações na estrutura genotípica), de forma a compreender os efeitos da adaptação a novas condições ambientais (e.g. temperaturas mais altas), e de que forma isso influenciará respostas a agentes de stress relacionados ou não com mudanças climáticas. Além disso, seria também interessante avaliar: i) o desfecho competitivo entre genótipos extremos, quando previamente aclimatados às condições de exposição; ii) se a adaptação dos organismos às flutuações ambientais seria suficiente para impedir a colonização de determinados *habitats* por espécies não nativas. Por outro lado, considerando que os professores são atores sociais fundamentais na mediação entre conhecimentos produzidos pelas comunidades científicas e comunidades educativas, particularmente alunos dos ensinos básico e secundário, seria importante desenvolver intervenções em formação de professores de ciências, inicial e contínua, visando estimular a sua participação em projetos de investigação, relevantes para o seu desenvolvimento pessoal e profissional, monitorizando-os e avaliando-os. Assim, importaria identificar pontos de vistas de professores de ciências e de formadores destes professores acerca de necessidades a este nível, por exemplo desenvolvendo instrumentos (*e.g.* questionários) destinados à realização de inquéritos.

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