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Growth, regeneration and colonisation of *Egeria densa* fragments*:* the effect of autumn temperature increases.

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Abstract:

The present study analysed the influence of higher temperatures on the growth, regeneration and colonisation abilities of apical shoot fragments from three naturalized and one cultivated population of *Egeria densa*. Our hypotheses were that (1) increased temperatures would favour the growth, regeneration and colonisation of *E. densa* shoots and (2) fragments from naturalized populations would have higher establishment success than fragments from cultivated plants. We tested the effect of average minimal autumn temperature (9°C), average maximal autumn temperature (16°C) and an increase of 3°C above these values, on apical shoots of these four populations of *E. densa* under controlled conditions in two growth chambers. Our results showed that temperature and the origin of the population had an effect on the growth rate of *E. densa* fragments, on their regeneration and colonisation abilities at the maximal autumn temperature. An increase of 3°C stimulated the growth rate of *E. densa* at low temperatures but had no effect on the plant colonisation and regeneration abilities. The responses of populations to low temperatures (9-12°C) were more similar than expected. In contrast, at higher temperatures (16-19°C) the cultivated population showed lower apical growth, higher regeneration and similar colonisation abilities to the naturalized populations. At these higher temperatures, the responses also differed among the naturalized populations. These results suggest that global warming has implications for the invasiveness of *E. densa*.

Keywords: invasive macrophytes; global warming; establishment success; functional traits.

Introduction

Climate change, variability and changes in land use are major drivers of ecosystem alterations. Non Indigenous Species (NIS) also contribute to ecosystem changes. They cause environmental damage, and have economic consequences and impacts on human-health. Interactions exist between these threats and they could exacerbate the impacts of climate change on ecosystems by changing environmental conditions, and climate change may also enable further invasions (Hellmann et al. 2008; Mainka and Howard 2010). Climate change could also affect the dynamics of plant invasions by favouring individual traits of invasive species (Hellmann et al. 2008). Aquatic non indigenous plants could benefit from the increasing seasonality and more marked wet and dry cycles.

In addition to physical changes, climate change can alter ecosystems and species' life cycles (Fitter et al. 1995; Bradley et al. 1999). Scientists have traditionally focused their attention on the seasonal changes in spring rather than those later in the year. Many studies have focused on spring events in temperate regions, while studies on the basic triggers of autumnal plant changes are, by comparison, in their infancy. Gallinat et al. (2015) consider that it is important to investigate the role of autumn climate change on NIS. The survival of invasive aquatic macrophytes in ecosystems depends, amongst other factors, on their thermal acclimation. Phenotypic plasticity and rapid evolution allow some invasive species to be more responsive to warmer autumn temperatures and to later freezing events than many native species (Richards et al. 2006), but there is a gap in our knowledge regarding invasive aquatic plant establishment and its responses to predicted climate change.

Among the invasive macrophyte species, *Egeria densa* Planch (Hydrocharitaceae), has been introduced worldwide by aquarium trade. Hence, *E. densa* has been included in the database of global invasive species (GISD 2015), by the Invasive Species Specialist Group (ISSG) of the Species Survival Commission of the International Union for Conservation of

Nature (IUCN)-World Conservation Union, although it is not in their top 100 list (Curt et al. 2010). *E. densa* is a perennial dioecious aquatic plant native from South America, in the neotropical range (Yarrow et al. 2009). In its introduced range, in Europe and in the United States, only male flowers have been observed, thus plants reproduce asexually by fragmentation (Cook and Urmi-König 1984). The large stands often seen in the US, Canada, New Zealand, Japan and Europe are considered to be genetic monocultures in each of these countries because of this sole reproduction strategy (Kadono et al. 1997; Darrin 2009). *E. densa* was introduced to France in the 1920s as experimental botanical material (St John 1961). These plants were released into the wild and have become naturalized in France, where their distribution has spread (Feuillade 1961a; 1961b). *E. densa* is found in both lentic and lotic environments (Yarrow et al. 2009) and it appears to be confined to warm-temperate and cool subtropical conditions. Within subtropical and tropical areas, *E. densa* is limited to high altitudes or cold-water springs (Cook and Urmi-König 1984). It is well adapted to cold climates and can survive freezing conditions during the winter by storing starch in its leaves and stems. It then uses these supplies for growth once temperatures rise above 10°C. It can even live for a while under ice (Cook and Urmi-König 1984). The optimum growth temperature reported is 16°C and the maximum temperature for growth is 25°C (Cook and Urmi-König 1984). Its growth is affected by temperature (Barko and Smart 1981; Riis et al. 2012; Hussner et al. 2014) but not by CO_2 availability under experimental conditions (Hussner et al. 2014). *E. densa* biomass has two growth maxima in August and December-January in Japan (Haramoto and Ikusima 1988) and in late summer and late autumn in South Carolina (Getsinger and Dillon 1984).

During the introduction phase, non-native aquatic species are usually introduced as stem fragments. Vegetative propagules such as stem fragments, are essential for the dispersal and colonisation of Hydrocharitaceae species (Silveira et al. 2009). The colonization of a new site by plant fragments requires regeneration (the ability of fragments to grow new shoots and

rhizomes), and establishment (the ability of fragments to develop roots and become attached to the sediment) (Barrat-Segretain et al. 1998; Riis et al. 2009). Although abiotic variables may also influence the survival and colonisation success of plant fragments, their effects on vegetative propagules are little known. In the Upper Parana River basin, physical and chemical properties of the water seem to be related to sediment characteristics, and plant fragments reaching habitats with different physical and chemical properties may differ in their colonisation and growth success (Silveira et al. 2009). The spread of *E. densa* is a range expansion from established populations via dispersal by water or via unintentional introduction by humans (emptying aquariums, propagation of fragments attached to boats, etc.). The present study analysed the influence of increasing autumn temperatures on the growth, regeneration and colonisation abilities of *E. densa* shoots from different populations during the first stage of the introduction process. We tested four populations: three "naturalized" populations and one "cultivated" population (from an aquarium supply store). Our hypotheses were that (1) an increase of three degrees would favour growth, regeneration and colonisation of *E. densa* fragments and (2) naturalized populations would have higher establishment success than a cultivated population.

Methods

Experimental design

Three sites A (N47°44'44"; W02°15'05"), B (N47°41'20"; W01°55'37") and C (N47°37'39"; W01°51'35") characterized by the presence of dense monospecific beds of *E. densa* were selected in Brittany, France. To characterize the environmental conditions in which *E. densa* was growing, we assessed the water quality of each site over the course of one year. At each sampling site, a water sample was collected in May, June, July, October and November 2013. Water temperature and the percentage of dissolved oxygen were measured in the field, whereas other parameters such as pH, conductivity and nutrients (PO_4^{3-}, NH_4^+, $N0_3^-$) were analysed in

the laboratory. Conductivity and pH were measured using a combined glass electrode and corrected for temperature (25°C). Reactive soluble phosphorus, nitrates and ammonia were analysed using a spectrophotometer (by the standard molybdenum blue analytical technique for phosphates and by the indophenol technique for ammonia, AFNOR, 1990).

In autumn 2013, forty shoots of *E. densa* were collected from each site (populations A, B and C) and forty shoots of *E. densa* were collected from a grower in the aquarium trade (population D). Shoots of population D had been grown at 26°C in the store.

Two experiments were carried out in autumn 2013. Climate change predictions for northern latitudes suggest an average temperature increase of around 3°C over the course of this century (McKee et al. 2003). To keep our experiment in line with these predictions, we subjected the plants from the four populations to two experiments with a temperature increase of 3°C. Experiment 1 consisted of testing the average minimal autumn temperature (9°C) and a temperature 3°C above this value, i.e. 12°C. In experiment 2, we tested the average maximal autumn temperature (16°C), and warmer conditions of 19°C.

Each experiment was set up under controlled conditions in two growth chambers (Percival AR-41L3X and Percival AR-41L3X LT). Water temperatures were maintained at specified levels (±1°C) using a temperature regulated chamber possessing both heating and cooling capacities. At the beginning of the experiment, the shoots from each population of *E. densa* were cleaned gently by hand to remove invertebrates, algae and debris. To each transparent plastic container (with dimensions L x W x H: 8 cm x 8 cm x 20 cm), we added sediment composed of 1 cm of substrate (potting soil) overlaid with 1cm of sand. Containers were filled with 500ml of tap water ($[NH_4^+–N]$ = 0.030 mg/l; $[NO_3^-–N]$ = 5.77 mg/l; $[PO_4^{3+}–P]$ = 0.010 mg/l; pH = 8.17; conductivity: 486 µS/cm). A 10 cm-long apical shoot (with green leaves and without roots, lateral shoots or flowers) was put into each plastic container. There were 10 replicates for each population and experimental temperature. The fragments grew

under 330 µmol photons m^{-2} s^{-1} as *E. densa* is reported to have optimum growth under these light conditions (Riis et al. 2012) in a 12/12 h light/dark cycle. The containers had randomly assigned positions in the growth chambers.

The impact of increasing temperatures on the growth of the fragments of *E. densa* was examined at the four temperature levels (9°C and 12°C, 16°C and 19°C) after 10 days of exposure, when the experiments were concluded. Pistori et al. (2004) showed that the Relative Growth Rate of *E. densa* was the highest between 8 to 12 days after the beginning of an experiment.

We measured 9 morphological traits at the end of the experiment: stem length (distance from shoot base to the shoot apex), internode length (mean value of ten internodes located 3-cm below the apex), number of buds, roots, flowers and lateral shoots. Lateral shoots included any secondary lateral shoots which developed from nodes of the original apical shoot and other lateral shoots that developed either from the same nodes, or from nodes of the lateral shoots (Di Nino et al. 2007). We also measured the length of lateral shoots (mean value), the mean root length and the area of a leaf located below the three-cm long apex. The difference between the stem length at the beginning and the end of the experiment indicated the relative growth rate "RGR" (Silveira et al. 2009).

$$\mathrm{RGR} = \frac{(\ln \mathrm{L2} - \ln \mathrm{L1})}{(\mathrm{t2} - \mathrm{t1})}$$

L1 and L2 refer to total length at times 1 and 2.

Statistical analyses

The normal distribution of the values and homogeneity of variance were checked, and a two–way ANOVA was performed to test the temperature and the population effects in experiments 1 and 2. For each significant difference ($p < 0.05$), a HSD Tukey's post-hoc test was performed to assign these differences between treatments. All data were analysed with Statistica 10.

Results

Plants were collected in neutral waters with high nutrient contents, moderate conductivity and low oxygen levels (Table 1). Site A was characterized by lower conductivity and ammonium concentrations than sites B and C (Table 1).

There was a significant interaction between temperature and population for bud and flower production under the minimal temperature conditions (Figure 1, Table 2), whereas there was no flower production at maximal autumn temperatures regardless of the population source. The growth (RGR), regeneration (production of lateral branches) and colonisation abilities (length of the roots) of *E. densa* fragments depended both on the population and temperature at the maximal autumn temperatures (Figure 1, Table 3). An increase of 3°C did not affect the production of buds, internode length or leaf area in any of the experiments. However, an increase of 3°C did have a significant effect on the apical growth (RGR) of *E. densa* at the minimal temperatures (experiment 1, 9-12°C), and on the regeneration abilities (production of lateral branches) at the maximal autumn temperatures (Figure 2; Tables 2, 3). The temperature increase from 16°C to 19°C also favoured the colonisation abilities (production of *E. densa* roots and their growth), whereas no roots or lateral branches were produced at 9°C and 12°C (Figure 2).

A significant population effect was established for all of the traits that were measured in both experiments, except for the RGR at minimal temperatures. Fragments from population A were characterized by lower growth (RGR and internode length), reduced leaf area and lower root development (number and length) than those of population C, and lower lateral shoots than those of the cultivated population (D) at maximal autumn temperatures (Figure 3). Fragments from population B had the highest leaf area in both experiments and developed high apical growth rates (RGR, internode length) and root production at maximal autumn temperatures (Figure 3). Fragments from population C allocated their energy to growth (the highest RGR) at

183 the maximal autumn temperatures (Figure 3). The fragments from the cultivated population D
184 had lower growth (lowest RGR at 16°C) and higher regeneration abilities (number of lateral
185 shoots) than the naturalized populations at maximal autumn temperatures (Figure 3). This
186 population was characterized by low leaf area (the lowest at minimal temperatures and among
187 the lowest at maximal autumn temperatures). Population D was the only one which produced
188 flowers at minimal temperatures (Figure 3).

189 **Discussion**

190 Our preliminary results showed that temperature and the origin of the population both
191 had an effect on the growth of *E. densa* (RGR*),* its regeneration (production of lateral branches)
192 and its colonisation (the length of the roots) abilities at the maximal autumn temperatures and
193 also on the production of buds and flowers at minimal autumn temperatures. An increase of
194 3°C at low temperatures slightly favoured the apical growth (RGR) of *E. densa*, whereas an
195 increase in temperature from 16°C to 19°C stimulated the regeneration and colonisation
196 abilities (number and length of roots). Our first hypothesis is supported.

197 We also demonstrated that the potential success of *E. densa* fragments to establish was
198 strongly influenced by the origin of the population. Fragments of *E. densa* from naturalized
199 populations elongated much faster than those from the cultivated population. Although the three
200 naturalized populations were acclimatized in Brittany, they showed different responses to
201 increasing temperature: fragments from population A had low growth, regeneration and
202 colonisation abilities, whereas the fragments from populations B and C had high regeneration
203 abilities and growth rates. Our results must be interpreted conservatively because differences
204 between the traits of fragments from populations A, B and C could be due to “ecotypes” or
205 “clonal adaptation”. The cultivated population had lower growth than the naturalized
206 populations when submitted to autumn temperatures: fragments allocated their energy

essentially to the production of lateral shoots (regeneration). Our second hypothesis is also supported.

We found very low growth of *E. densa* at 9°C, but significantly higher growth when the temperature increased to 12°C. The increase in temperature from 16°C to 19°C stimulated the production of lateral branches amongst population D and the apical growth of fragments from all populations. These results are congruent with data in the literature (Getsinger and Dillon 1984; Haramoto and Ikusima 1988; Hussner et al. 2014). Haramoto and Ikusima (1988) showed that below 10°C, the growth of *E. densa* stops. Similarly, the autumn biomass peak was recorded when the water temperature had reached approximately 10°C in Lake Marion in South Carolina (Getsinger and Dillon 1984). Haramoto and Ikusima (1988) also showed that lateral shoots with roots were developed and elongated towards the water surface when the water temperature rose above 15°C, and that optimum growth rate occurred at a temperature of 20.7°C. We measured the highest RGR (0.032 day^{-1}) for population C at 19°C and the lowest for population A at 19°C (RGR = 0.018 day^{-1}). According to laboratory findings from other studies, the average measured RGR of fragments from naturalized populations of *E. densa* is consistent with the data reported in the literature for that temperature range (Barko and Smart 1981; Haramoto and Ikusima 1988; Tanner et al. 1993; Carrillo et al. 2006; Silveira et al. 2009; Hussner et al. 2014).

In this study, we showed that the high capacity of *E. densa* populations to adjust their growth responses depends on both the environmental temperature conditions and the origin of the plants. The acclimation potential towards cold temperatures may be closely related to the temperatures experienced in the area where plants were collected. Indeed, the responses of the three naturalized populations of *E. densa* when acclimated to 9°C and 12°C were similar, and differed significantly from the cultivated population for the number of flowers, internode length and leaf area. All naturalized populations may have partly entered into a dormant stage due to

232 the minimal temperatures, as seen in other studies (Madsen and Brix 1997; Hyldgaard et al.
233 2014). This possibility is supported by observed morphological changes, such as shoots of the
234 naturalized plants tested at 9°C and 12 C, which were less compact in structure and had longer
235 distances between whorls (mean internode length = 0.36 ±0.07 cm) than the plants exposed to
236 16°C and 19°C (mean internode length = 0.50 ±0.17cm). In contrast, the fragments from the
237 cultivated population produced flowers and buds when they were exposed to the low
238 temperatures.

239 The three naturalized populations were collected from neighbouring sites in Brittany
240 and therefore they had been exposed to the same climatic conditions prior to the experiments.
241 The environmental conditions in the field could explain some differences in the responses
242 between the three naturalized populations of *E. densa* tested. Feijoó et al. (2002) found that *E.*
243 *densa* shows a clear preference for ammonium over nitrate and that most nitrogen was absorbed
244 from the water column. Moreover *E. densa* can store nutrients in their leaves and stems. The
245 comparatively high concentration of ammonium in sites B and C could suggest that nitrogen
246 was stored in the plants and used later for growth (luxury consumption hypothesis). This could
247 explain the higher growth rate of *E. densa* from the sites B and C. Furthermore, the RGR is
248 directly controlled by the concentration of N within the plant (Ågren 1985) and high
249 concentrations of N in the tissues may favour plant growth.

250 In Western Europe, as only male plants and clonal reproduction have been observed, the
251 wide range of morphological variations within *E. densa* species suggest that either a large
252 number of diverse clonal lineages were introduced, or that this plant has high levels of somatic
253 mutation. For functional traits such as growth rate, phenotypic plasticity can allow invasive
254 plants to benefit from changing environmental conditions (Richards et al. 2006). Morphological
255 plasticity may evolve rapidly in introduced species, and thereby contribute to their spread and
256 invasion success after a time-lag (e.g. Sexton et al. 2002). Results of the present study

confirmed that *E. densa* could act aggressively as an invasive plant by showing plasticity for ecological traits that are beneficial to its range expansion. At this time, we do not have information about potential genetic differences between the different populations of *E. densa*. Our hypothesis should be tested by genetic approaches.

Our preliminary results suggest that *E. densa* may be able to develop taller canopies in the future, in particular in autumn, which might improve their survival during the winter and accelerate their growth in early spring. To test our hypothesis, it would be necessary to assess the responses of these different populations of *E. densa* during a long term study. We emphasize that our observations were recorded in the early stages of fragment regeneration, and whether they also pertain to later life stages is a matter for further investigation. Moreover, *E. densa* has the ability to adapt to seasonal changes in temperature in its natural habitat (Haramoto and Ikusima 1988). The growth rate of *E. densa* in autumn will be sensitive to the increase of temperatures expected in the context of global warming. These results should be tested in the field. The present study has investigated the growth responses of *E. densa* to varying temperatures, in isolation from a number of other environmental factors which would operate in the field (e.g. light, macrophyte beds, disturbance, nutrients, water velocity, etc). Research on the response of this species to increasing temperatures in parallel to other environmental factors is required to improve our understanding of the impact of global warming on the invasiveness of aquatic plants.

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sites	Temperature			pH			Conductivity			N- NO_3-			P-PO_4^{3-}			N- NH_4+			O_2		
	°C						$\mu S.cm^{-1}$			$mg.L^{-1}$			$\mu g.L^{-1}$			$\mu g.L^{-1}$			%		
A	16.9	±	8.2	7.135	±	1.3	227.5	±	15.2	4.41	±	0.7	0.105	±	0.1	0.375	±	0.1	29	±	20
B	16.3	±	5.9	7.415	±	0.9	415.7	±	60	3.53	±	3.2	0.356	±	0.4	0.992	±	0.5	34	±	1.7
C	15.6	±	4.5	7.958	±	1,0	345.7	±	13.3	3.92	±	3.5	0.118	±	0.1	0.977	±	0.5	27	±	4.9

Table 1: Water chemical composition of the three sampling sites (annual mean value, n = 4).

	Degree of freedom	F	p
Relative Growth Rate			
population	3	1.51	0.22
temperature	1	4.60	**0.04**
population x temperature	3	1.83	0.15
Number of buds			
population	3	6.28	**0.0068**
temperature	1	0.56	0.14
population x temperature	3	3.09	**0.025**
Internode length			
population	3	46.87	**< 0.0001**
temperature	1	0.13	0.72
population x temperature	3	0.56	0.65
Leaf area			
population	3	30.20	**< 0.0001**
temperature	1	0.13	0.72
population x temperature	3	0.30	0.83
Number of flowers			
population	3	6.95	**0.0004**
temperature	1	6.70	0.01
population x temperature	3	6.95	**0.0004**

372

373 Table 2: F-Values and significance of two-way ANOVA of the effects of population and
374 temperature on growth and morphology parameters of Egeria densa submitted to minimal
375 autumn temperature (Experiment 1, 9-12°C).

376

Relative Growth Rate	Degree of freedom	F	p
population	3	17.30	**< 0.0001**
temperature	1	2.55	0.11
population x temperature	3	3.66	**0.016**
Internode length			
population	3	5.11	**0.003**
temperature	1	0.17	0.68
population x temperature	3	1.22	0.31
Number of Buds			
population	3	6.28	**0.0008**
temperature	1	0.56	0.46
population x temperature	3	3.09	**0.03**
Number of lateral branches			
population	3	18.56	**< 0.0001**
temperature	1	4.64	**0.03**
population x temperature	3	3.15	**0.03**
Length of Lateral Shoots			
population	3	6.45	**0.0006**
temperature	1	1.73	0.19
population x temperature	3	1.92	0.13
Number of Roots			
population	3	4.78	**0.004**
temperature	1	10.19	**0.002**
population x temperature	3	2.02	0.12
Length of roots			
population	3	7.30	**0.0002**
temperature	1	7.26	**0.009**
population x temperature	3	3.93	**0.01**
Leaf area			
population	3	10.29	**< 0.0001**
temperature	1	0.06	0.80
population x temperature	3	0.85	0.47

Table 3: F-Values and significance of two-way ANOVA of the effects of population and temperature on growth and morphology parameters of Egeria densa submitted to maximal autumn temperature (Experiment 2, 16-19°C).

Figure captions

Figure 1: Comparison of the effect of population and temperature on bud number at 9-12°C (experiment 1) and on Relative Growth Rate (RGR) at 16-19°C (experiment 2) between *E. densa* populations. Different letters indicate significant differences for *E. densa* with temperature and population (ANOVA, $p < 0.05$). Bars indicate standard deviation.

Figure 2: Comparison of the significant effects of temperature on traits between *E. densa* populations in experiments 1 and 2. Different letters indicate significant differences for *E. densa* with temperature (ANOVA, $p < 0.05$). Bars indicate standard deviation.

Figure 3: Comparison of the significant effects of population on traits between *E. densa* populations in experiments 1 and 2. Different letters indicate significant differences for *E. densa* with population according to ANOVA, $p < 0.05$ and Tukey test. Bars indicate standard deviation.