PRIMARY RESEARCH PAPER

Invasive Australian swamp stonecrop (*Crassula helmsii*) negatively affects spawning but accelerates larval growth of the endangered natterjack toad (*Epidalea calamita*)

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Abstract The invasive *Crassula helmsii* is rapidly expanding in Europe. Several ecological effects are described, most of which focus on ecosystem functioning and native vegetation but rarely on fauna. In North-western Europe, *C. helmsii* often invades the breeding habitat of endangered amphibians, such as *Epidalea calamita.* The spawning rate, egg survival and the speed of larval growth of this toad species in relation to the presence of *C. helmsii* were studied. In order to unravel causal mechanisms, effects on natterjack toads were related to the effects of *C. helmsii* presence/absence on temperature and chemical properties of the water. Spawning and egg survival were significantly lower under *C. helmsii* dominance compared to bare soil conditions, and negatively affected the population size of *E. calamita*. However, larval growth rate was significantly higher in *C. helmsii* dominated treatments, which could be beneficial. Differences in water temperature and chemistry were a possible explanation for these effects. It remains unclear whether the population viability of *E. calamita* is negatively affected when *C. helmsii* is present. In many areas, however, this plant species completely

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overgrows and causes desiccation of waterbodies. Therefore, appropriate management measures will be required to protect this toad against this invader.

Keywords Alien plant invasion · Amphibious weed · Species interactions · Threatened amphibians

Introduction

Invasive alien species are considered to be a major threat to world's biodiversity (e.g., Hobbs, 2000; McNeely, 2001; Mooney & Cleland, 2001; Clavero & García-Berthou, 2005; Genovesi, 2005; Butchart et al., 2010; Ruckelshaus, 2020). The processes of competition and niche displacement, among others, can eventually result in the local extinctions of populations of endangered species (Mooney & Cleland, 2001; Genovesi, 2005; Blackburn et al., 2019; Rabitsch et al., 2020). The Australian swamp stonecrop *Crassula helmsii* (T. Kirk) Cockayne, native to Australia and New Zealand, has a high dispersal ability and is regarded as an invasive species in North-western Europe owing to its ability alter habitats through extremely high annual biomass production (Dawson & Warman, 1987; OEPP/EPPO, 2007; Smith and Buckley, 2020). This perennial amphibious weed is increasingly dominating moorlands, dune slacks, lakes and other freshwater to slightly brackish wetlands, thereby threatening protected ecosystems and endangered species (Dawson & Warman, 1987; Leach & Dawson, 1999; OEPP/EPPO, 2007; Newman, 2013; Brouwer et al., 2017; Prinz et al., 2019; Smith & Buckley, 2020). A variety of habitat alterations after invasions by *C. helmsii* have been described, such as changes in hydrology and water quality (Newman, 2013), reduction of characteristic species richness in aquatic ecosystems (Dawson & Warman 1987; Leach & Dawson, 1999; Hussner, 2009) and effects on plant species (Pilkington, 2016). Other studies concluded that *C. helmsii* had impacts on biodiversity similar to fast growing native species such as *Phragmites australis* (Cav.) Trin. ex Steud. (Dean, 2015), whereas in a field study in England no species loss or germination suppression of native plant species was observed (Langdon, 2004). Several studies have described positive effects of invasive alien species, such as providing shelter for invertebrates, tadpoles or increasing the food supply (e.g.

Rodriguez, 2006; Grutters et al., 2015; Reyne et al., 2021; Velle et al., 2022). However, such positive effects of the species have not yet been described for *C. helmsii,* with the exception of Smith & Buckley (2015) who found that *C. helmsii* increases the rarity scores of macrophyte assemblages in south-east England. As noticed for other aquatic weeds, *C. helmsii* may contribute to production and regulating ecosystem services. In various European countries, this species is still sold as an aquarium and pond plant (Dawson & Warman, 1987; OEPP/EPPO, 2007). In Europe, the flowering period lasts from June to the end of October. It has been suggested that these flowers may be a source of nectar for insects (Lockton, 2009). Outside of its native range, however, no pollinators have as yet been observed (Dawson & Warman, 1987; Smith & Buckley, 2020).

In North-western Europe*, C. helmsii* often invades the breeding habitat of several endangered species, such as amphibians (OEPP/EPPO, 2007). Few studies explicitly examined relationships between *C. helmsii* and animals. Egg-hatching of the newt *Lissotriton vulgaris* (L., 1758) was delayed on *C. helmsii* when compared to their preferred native species *Nasturtium officinale* R.Br. (Langdon, 2004). Diaz (2012) suggested that aquatic invertebrates and fish species were negatively affected by depletion of CO_2 in the water column caused by *C. helmsii*. In contrast to these findings, several studies also reported no effect of *C. helmsii* invasions on macroinvertebrate diversity (Ewald, 2014; Smith, 2015; Smith & Buckley, 2015). However, knowledge about the effects of *C. helmsii* is lacking for many endangered species.

Populations of the natterjack toad *Epidalea calamita* (Laurenti, 1768) may be threatened by *C. helmsii* invasions. This endangered amphibian species and its habitats are protected according to the European Habitat Directive Annex IV (Council of the European Communities, 1992). In the Netherlands, their populations have declined by 40% since 1950 (Goverse, 2009; Netherlands Enterprise Agency, 2014), and the species has been added to the Dutch Red List of Endangered Species (Ministry of Agriculture, Nature and Food Quality, 2009). The IUCN 3.1 Red List Assessment of *E. calamita* also recognized that populations are declining, but classified the species as least concern (Beja et al., 2009).

In this paper, the effects of *C. helmsii* dominance on the reproduction and larval growth of *E. calamita*

were examined using field surveys and an enclosure experiment. Species interactions are expected as both species occupy similar pioneer habitats, characterized as edges of temporary water bodies with gentle slopes, consisting of sandy soils with open patches in vegetation and low plant litter (Beebee, 1979; Dawson & Warman, 1987; Sinsch, 1998; Creemers et al., 2009; Brouwer et al., 2017; Rayne et al., 2021; Van der Loop et al., 2020). Invasions of *C. helmsii* could affect the suitability of habitats for the reproduction of *E. calamita* through overgrowing sparsely vegetated soils that are used for egg deposition by this amphibian. As *C. helmsii* fills up the water column, competition for space increases, which is known to reduce the survival rate of tadpoles (Brady & Griffiths, 2000). Dominant presence of *C. helmsii* can also profoundly affect water temperature and chemistry, thus possibly affecting microclimate, water quality and/or food availability for *E. calamita* tadpoles. Compared to sparsely vegetated littoral zones and inundated banks, these bare soil breeding habitats invaded by *C. helmsii* are expected to show less strong temperature fluctuations due to shading effects at daytime and insulating effects at night time, possibly affecting hatching of eggs as well as speed of larval growth. *C. helmsii* is also known to absorb a substantial amount of nutrients from the water layer, possibly reducing the productivity of larvae-preferred algae (Newman, 2013; Van Kleef et al., 2017), whereas the succulent vegetative parts of this plant are expected to be inedible to tadpoles. When less food is provided, tadpoles could exhibit a slower growth rate and lower chance of survival (Golay, 1996; Sinsch, 1998). Tadpoles are at increased risk of not reaching metamorphosis before desiccation of pools and rising of dissolved organic substances result in deterioration of water quality, or *C. helmsii's* high biomass production leaves insufficient movement space for swimming organisms, such as tadpoles, probably resulting in problems with resource acquisition (Sinsch, 1998). Malnourished tadpoles remain smaller as juveniles generally resulting in a lower survival rate on land, since smaller juveniles dehydrate more quickly and are slower, increasing the risk of predation (Altwegg & Reyer, 2003; Reques & Tejedo, 1997). The dominant presence of *C. helmsii* can also result in lowered pH and changes in water chemistry (Newman, 2013; Van Kleef et al., 2017). Such acidification may lead to lethal toxic ammonium and aluminium stress in *E.*

calamita tadpoles (Leuven et al., 1986; Fedorenkova et al., 2012).

In order to study the effects of *C. helmsii* invasion on the reproductive success of *E. calamita*, field surveys and an enclosure experiment were performed at a breeding site of this toad. We surveyed the spawning of *E. calamita* at soils dominated by *C. helmsii* and at bare soils. An in situ enclosure experiment was executed to determine the effect of *C. helmsii* on egg-hatching and early larval growth. We hypothesized that *C. helmsii* invasion affects *E. calamita*'s breeding success through:

(i) reduced spawning (egg deposition) due to the complete overgrowth of its spawning habitat (i.e. bare soils in littoral zones of shallow and temporary waterbodies);

(ii) reduced larval growth resulting from nutrient uptake by plants from the water thereby reducing growth of algae, which are the preferred larval food source;

(iii) reduced larval growth due to alteration of water chemistry resulting in a toxic environment for tadpoles;

(iv) reduced larval growth due to reflection of sunlight and reduced temperature fluctuation.

Materials and methods

Study site

The field surveys and enclosure experiments were conducted in the nature area 'De Gijzenrooise Zegge' in the province of Noord-Brabant, the Netherlands (51° 24′ 24.29″ N, 5° 31′ 36.87″ E). This area is characterized by a brooklet flowing through wet grasslands with fens and natural lakes. Recently, the ground water level of the study site has been artificially increased in order to mitigate effects of desiccation. The area is designated as a nature conservation site since it harbours a population of *E. calamita*. The study site consists of a seasonally inundated area containing a small, shallow fen-like lake of 0.75 ha, fed by rainwater and characterized by sparse vegetation. The waterbody has been restored, and is managed, to maintain in a low succession phase containing sparsely vegetated and bare soils which are used by

E. calamita for reproduction (Fig. 1). Approximately 5 to 6 years ago, *C. helmsii* invaded this site. Due to absence of native vegetation, the species could form dense swards on a large part of the waterbody and its banks creating a situation with *C. helmsii* dominance alternating with bare soil. Expansion of the plant to bare soils is ongoing with the expectation that eventually these waterbodies will become totally overgrown by the invasive species.

Experimental setup

Field surveys of vegetation and spawning activity

To determine if *C. helmsii* cover affected spawning of *E. calamita*, spawn string release was surveyed immediately after the peak of spawning activity, along a 200 m transect on the west bank of the lake in the 'De Gijzenrooise Zegge'. Here, spawn strings were observed in previous years (pers. observation M. van de Loo). Every 5 m along the same 200 m transect,

the cover of *C. helmsii* and bare mineral soils were estimated in percentages on the 9th of April 2018, in the upper 3 m of the littoral zone. In addition, all spawn strings of *E. calamita* were counted along this transect in order to characterize spawning preference in relation to vegetation cover. Within a 2 m radius of each recorded spawn string, the cover (%) of *C. helmsii* was recorded as well.

Enclosures

On the 9th of April 2018, 40 enclosures were placed in a transect parallel to the shore of the natural lake in the 'De Gijzenrooise Zegge' (Table 1). The area in which all enclosures were placed was 0.04 ha and each enclosure was located at a maximum distance of 5 m from other enclosures, in order to control for site effects. The enclosures consisted of a black PVC tub without a bottom. These tubs had a semi-cylindrical shape with a diameter of 53 cm at the widest part, and 48 cm at the narrowest part (in total ca. 56.13 l). All

Fig. 1 **A** Management of breeding sites for *Epidalea calamita* is aimed at maintaining a low vegetation cover. **B** The rapid growth of the invasive species *Crassula helmsii* can result in a complete vegetation cover, and the loss of surface water within a few years

Table 1 Experimental set-up for effect assessment of *Crassula helmsii* on hatching success and larval development of *Epidalea calamita* in the nature area 'De Gijzenrooise Zegge'

Enclosure number	Soil	Containing *E. calamita* eggs	Water chemistry measurements	Temperature measurements
1–10	Bare sand	Yes	Yes	Yes
11–20	Bare sand	No	Yes	No
21–30	*C. helmsii* dominated	Yes	Yes	Yes
31–40	*C. helmsii* dominated	No	Yes	No
Outside enclosures				
Bare soils	Bare sand	Yes	No	Yes, at 2 locations
Vegetated soils	*C. helmsii* dominated	Yes	No	Yes, at 2 locations

tubs were 48 cm high, of which 20 cm was pressed into the soil to prevent the escape of tadpoles. In total, 20 enclosures were placed at soils with a vegetation that was dominated by *C. helmsii* (cover: 100%, average water depth 25.5 cm). The other 20 enclosures were placed on sparsely vegetated soils (native vegetation absent, cover of *C. helmsii* < 2%, average water depth 22 cm; hereafter: bare soil). At the start of the experiment, all potential predators (i.e. Anisoptera and Notonecta sp.) of *E. calamita* eggs and tadpoles were removed from the enclosures. The top of the enclosures was covered with woven net with a mesh size of 2 × 2 cm to prevent predation of tadpoles by birds. Eggs of *E. calamita* were incubated in 10 enclosures on bare soil and 10 on *C. helmsii* dominated soil. The remaining 10 enclosures on bare soil and 10 on *C. helmsii* dominated soil were not incubated with eggs. This experimental set-up allowed comparisons of water chemistry and temperature in enclosures with and without 100 eggs per enclosure.

Egg hatching

On the 10th of April 2018, circa 2.000 eggs of *E. calamita* were collected at the study site from the same shallow lake. 100 eggs per enclosure were separated from the sting by hand (wearing latex gloves for protection of the eggs) and incubated in 10 enclosures with *C. helmsii* dominated soils and 10 on bare soil. The eggs in each enclosure originated from at least five strings to ensure sufficient genetic diversity of eggs and to compensate for possible differences in development time. After 10 days of incubation in the enclosures, eggs either hatched, resulting in a tadpole, or clearly failed to develop and died. The

number of successfully hatched eggs was determined in each enclosure by counting live tadpoles.

Larval growth

Larval growth following hatching was monitored for 43 days until the last tadpoles reached early metamorph stage. From each enclosure containing *E. calamita* tadpoles, 15 individuals were randomly caught once a week between 20th of April and 7th of June 2018, using a small landing net. Each captured tadpole was then transferred by hand, wearing powderless latex gloves in a water-filled cuvette. Subsequently, total length was measured for each individual by placing the cuvette on plasticized millimetre paper and taking a picture. This ensured an accurate measurement while minimizing disturbance for the tadpoles. However, this method did not allow for individual follow-up measurements of each tadpole. The data collected was a set of measured lengths of the respective enclosure's tadpole population. After each measurement, tadpoles were returned to their respective enclosure. To prevent metamorphosed toads from drowning in the enclosures, any tadpoles that reached early metamorphosis were released into their natural habitat outside the enclosures immediately after the last measurement.

Water chemistry

To determine effects of enclosures, presence of tadpoles and/or *C. helmsii* dominance on water chemistry, water samples were taken at the start of the experiment (16th of April 2018) and at three times during the experiment (24th of April, and 9th and 23rd May

2018) in all enclosures (with and without tadpoles present). The pH was measured with a standard combined glass Ag/AgCl pH electrode (Orion Research, Beverly, CA, USA) connected to a pH meter (Tim800; Radiometer analytical, Lyon, France). An auto-analyser 3 system (Bran & Lubbe, Norderstedt, Germany) was used to measure concentrations of nitrate (NO_3^-), ammonium (NH_4^+), sodium (Na^+), chloride (Cl^-) and aluminium (Al^{3+}) colourimetrically using hydrazine sulphate (Kamphake et al., 1967).

Water temperature

Water temperature was measured using HOBO Pendant UA-001-08 data loggers placed in all enclosures containing tadpoles. Water temperature was registered every 15 min from 14th of April at 12:00 AM to 20 of May at 11:00 PM. In order to evaluate the effect of the enclosures on water temperature, four additional data loggers were placed outside the enclosures, two on *C. helmsii* dominated soil and two on bare soils. For each day and each separate logger, we obtained mean temperature (T_{mean}), maximum (T_{max}) and minimum (T_{min}) recorded temperature and derived from these the maximum daily temperature fluctuation ($T_{fluc} = T_{max} - T_{min}$) and maximum daily fluctuation scaled by daily mean temperature ($T_{fluc/mean} = T_{fluc}/T_{mean}$).

Statistical analysis

All data were tested and visualized using the statistical programme R version 4.0.2 (R core Team 2020), using the packages lme4 (Bates et al., 2015), multcomp (Hothorn et al., 2008), AER (Kleiber & Zeileis, 2008), qpcR (Spiess, 2018), car (Fox & Weisberg, 2019), PMCMRplus package (Pohlert, 2021), Gamm4 (Wood & Scheipl, 2020), lawstat (Gastwirth et al., 2020), Hmisc (Harrell & Dupont, 2020) and mgcv (Wood, 2011). The R code and statistical output is added as supplementary material (SM) appendix I (online appendix to this paper).

Egg spawning

We tested whether mean *C. helmsii* cover significantly influenced number of egg spawn strings encountered using a General Linear Model (GLM) with negative binomial distribution, since data was discrete,

not normally distributed (Shapiro–Wilk: $W = 0.529$, P-value ≤ 0.001) and showed significant overdispersion in an a priori performed poisson GLM model (dispersion factor 40.06, $P < 0.001$).

Egg-survival

Normality assumptions were met for the number of hatched eggs in enclosures (Shapiro–Wilk: $W = 0.970$, p-value $= 0.759$). Therefore, effect of contrasting habitat structure categories (bare soil versus 100% cover of *C. helmsii*) on number of hatched eggs were tested using a Linear Model (LM).

Larval growth

Initial fitting of linear or generalized linear models on larval growth over time resulted in significant over- or underfitting of our measured data over the time period. We accounted for this by testing effects of contrasting habitat structure categories on larval growth using a Generalized Additive Model (GAM) approach, with the smoothing term modelling the time effect on larval size and the categorical variable (bare soil vs 100% *C. helmsii* dominance) as additive predictor variable affecting larval size (for model parameters, see SI Table 3). Apart from *C. helmsii* dominance vs bare soil treatment, the initial number of tadpoles, counted by successfully hatched eggs, at the start of the experiment was hypothesized to affect larval growth rate by competition between tadpoles. Consequently, the initial number of tadpoles was also incorporated in the analysis as a covariate.

We specified several models varying in degree of complexity, including a null-model, main treatments (*C. helmsii* dominating *vs* bare soils; both as additive and interacting factor) and the number of initial tadpoles at the start of the experiment (SI Table 4) and selected the best describing model using the respective model BICs. Since data were not normally distributed, continuous but increasing in variance over time, we performed a Gamma distributed GAM with identity link for each model.

Water chemistry analysis

Effects of *C. helmsii* dominance on water chemistry (pH, alkalinity and seven different water chemistry components, CO_2, HCO_3^-, NO_3^-, NH_4^+, Na^+,

Cl^- and Al^{3+}) were analysed. Assumptions for normality were met for pH (Shapiro–Wilk: $W = 0.991$, P-value $= 0.392$), HCO_3^- (Shapiro–Wilk with log transformation: $W = 0.991$, P-value $= 0.392$) and NH_4^+ (Shapiro–Wilk with log transformation: $W = 0.994$, P-value $= 0.820$). An ANOVA of a linear model was used to test for treatment effects. Normality assumptions were not met for: NO_3^-, (Shapiro–Wilk: $W = 0.631$, P-value < 0.001), Na^+ (Shapiro–Wilk: $W = 0.783$, P-value ≤ 0.001), Cl^- (Shapiro–Wilk: $W = 0.781$, P-value ≤ 0.001) and Al^{3+} (Shapiro–Wilk: $W = 0.565$, P-value < 0.001), and therefore a non-parametric Kruskal–Wallis test was used to test for these treatment effects.

Water temperature analysis

Since assumptions for normality of temperature data were not met, we tested for significance of differences between treatments using a Kruskal–Wallis non parametric test, and compared for statistical differences between treatment groups with the Dwass, Steel, Critchlow and Fligner procedure from the PMCMR-plus package (Pohlert, 2021).

Results

Egg spawning

During the field survey a total of 48 spawn strings were found in the littoral zone of the western part of the lake in 'De Gijzenrooise Zegge'. The mean cover (%) of *C. helmsii* in areas where *E. calamita* spawn strings were found was significantly lower than that in areas where spawns were absent (30.45% and 1.60% cover, respectively, Fig. 2, $z = 5.866$, $P < 0.001$, SI Table 1).

Egg-survival

The mean percentage of hatched eggs was significantly higher (LM: $t = -4.543$, $P \leq 0.001$ $N = 20$) in enclosures placed on bare soils (87.7%) in comparison to enclosures placed on *C. helmsii*-dominated soil (65.9%) (Fig. 3 and SI Table 2).
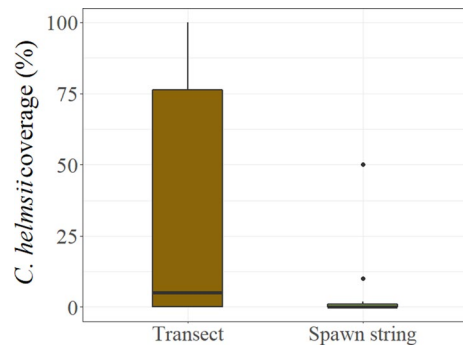

Fig. 2 Cover of *Crassula helmsii* (%) at locations along a 200 m transect ($N = 40$) and within a radius of 2 m around spawn strings of *Epidalea calamita* ($N = 48$) in a natural lake of 'De Gijzenrooise Zegge'. Boxplots display the first quartile (bottom line of box), median (middle line of box), third quartile (top line of box) and maximum (highest value). Dots indicate outliers in data
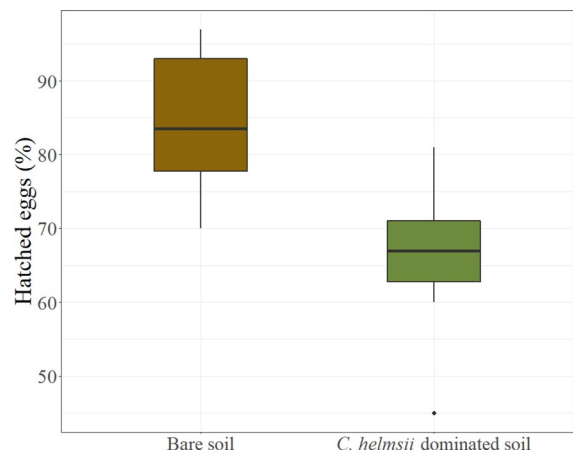

Fig. 3 Average hatching success of *Epidalea calamita* eggs (%) on bare mineral soils ($N = 10$) and *Crassula helmsii* dominated soils ($N = 10$) in field enclosures. Boxplots display the first quartile (bottom line of box), median (middle line of box), third quartile (top line of box) and maximum (highest value). Dots indicate outliers in data

Larval growth

Eggs started hatching on April 20th, and the first metamorphic toads were observed on May 22nd. When comparing all fitted GAM models, the model incorporating different smoothing curves for *C. helmsii* dominated and bare soil enclosures and including an additive effect of *C. helmsii* versus bare soil treatment as predictors of length by

development time received most support (SI Tables 3 and 4: Model 1; $W_i = 0.93$). All other models received little support (SI Table 3 and 4: Model 5 (included initial number of tadpoles as additive effect): rel. ll = 0.09; $W_i = 0.07$) or very low support (SI Tables 3 and 4: all other models specified: $W_i < 0.001$). Hence, model 1 was used in estimation of parameter effects (SI Tables 3 and 4).

Mean total length of tadpoles was significantly higher in the enclosures with *C. helmsii* dominated soil than in enclosures with bare soils (SI Table 5; *C. helmsii*: 14.51; Bare Soil: 13.69; Fig. 4, $t = -13.1$, $P \leq 0.001$). Average length of tadpoles increased significantly faster under conditions with *C. helmsii* dominance than with bare soil (see difference between smoothing curves in Fig. 4, calculated from the model parameters of model 1; Tables SI 3,4 and 5).

The development of tadpoles in enclosures dominated by *C. helmsii* was faster than in enclosures on bare soil (Fig. 4). The onset of metamorphosis of tadpoles in these enclosures started after day 28 and 31, respectively.
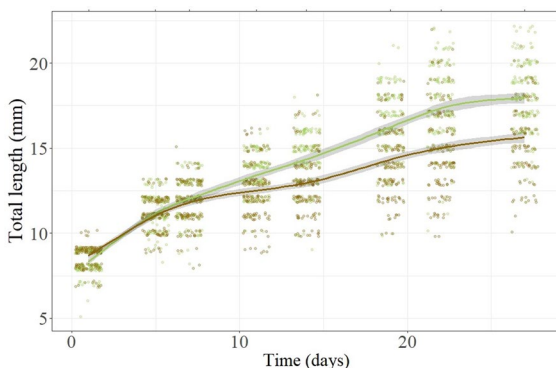

Fig. 4 The total length (mm) of individual *Epidalea calamita* tadpoles ($N = 15$; 8 measurements over a period of 28 days) on bare soil enclosures (indicated by brown colour, $N = 20$) and *Crassula helmsii* dominated soils (indicated by green colour, $N = 20$, $P \leq 0.001$). Lines indicate the average length of tadpoles. Shading around the lines show the 95% confidence intervals. Day 0 is first day of measuring, April 20th 2018. Measuring data was jittered along y and y axis to increase visibility of individual data points

Water chemistry

The pH of surface water significantly differed between the four different measuring rounds ($F = 17.904$, $P < 0.001$ Table SI_7), this was mainly caused by higher pH values during the last two measurements, and excluding these measurements resulted in no significant pH effect (for test results, see SI Table 9).

Furthermore, the pH was significantly lower in the *C. helmsii*-dominated enclosures (average 6.30, SE ± 0.02 in water on bare soil and 6.05, SE ± 0.02 in *C. helmsii* dominated enclosures $F = 107.780$, $P < 0.001$, SI Fig. 1, SI Tables 6, 7 and 9).

On the last two measuring dates, ammonium and nitrate concentrations were significantly lower in enclosures containing *C. helmsii* (respectively: $F = 16.120$, $P < 0.001$ and $\chi^2 = 110.600$, $P \leq 0.001$ SI Figs. 2 and 3, and SI Tables 6, 7, 8 and 10). No effect of presence of *E. calamita* eggs on this ammonium concentration was found ($F = 1.540$, $P = 0.217$). Water temperatures rose above 25 °C and stimulated the decomposition of organic matter and therefore the release of inorganic nitrogenous compounds. Elevated inorganic nitrogen levels were measured in bare soil enclosures on the last two measuring dates. In *C. helmsii* dominated enclosures this increase was not observed.

During the experiment, evaporation caused a decrease in water levels resulting in significantly increased concentrations of sodium, chloride and aluminium (respectively: $\chi^2 = 122.850$, $P < 0.001$, $\chi^2 = 131.740$, $P < 0.001$ and $\chi^2 = 98.705$, SI Tables 6 and 8). The enclosures with *C. helmsii* dominated soil contained more water in the later stages of the experiment, presumably resulting from lower mean water temperatures (see below) in these enclosures, which reduced evaporation and therefore limiting the concentration effect of evaporation (SI Figs. 4, 5 and 6).

Water temperature

Plots of temporal fluctuations of water temperature and differences between treatments during the experiment are added as supplementary material (online appendix to this paper). T_{mean} differed between treatments (Kruskal–Wallis rank sum test: $\chi^2 = 9.14$, $df = 3$, $P = 0.028$), but between-treatment differences were not significant (post-hoc test; $P > 0.05$ for all comparisons; SI Fig, 7A). T_{fluc} differed significantly

between treatments (Kruskal–Wallis rank sum test: $\chi^2 = 69.44$, $df = 3$, $P < 0.001$) and was significantly larger on bare soils outside the enclosures compared to bare soil and *C. helmsii* within the enclosures (SI Fig. 7, panel B; $P < 0.001$), but not between *C. helmsii* and bare soil locations outside enclosures (SI Fig. 7, panel B; $P > 0.05$). Within enclosure treatments, daily temperature fluctuations were significantly smaller in *C. helmsii* dominated enclosures compared to bare soil (SI Fig. 7, panel B; $P < 0.001$).

Maximum day-time temperature of water differed significantly between treatments (Kruskal–Wallis rank sum test: $\chi^2 = 23.2$, $df = 3$, $P < 0.001$), but only between the enclosures and sampling locations outside the enclosures, with higher maxima outside the enclosures (SI Fig. 7). Minimum day time temperature differed significantly between treatments (Kruskal–Wallis rank sum test: $\chi^2 = 16.4$, $df = 3$, $P < 0.001$), but only was significantly lower in *C. helmsii* dominated enclosures compared to bare soil enclosures (SI Fig. 7, panel C, $P < 0.001$).

Mean water temperature scaled daily fluctuation ($T_{fluc/mean}$) differed significantly between treatments (Kruskal–Wallis rank sum test: $\chi^2 = 73.8.4$, $df = 3$, $P < 0.001$) and was significantly higher at sites with bare soil outside enclosures compared to all other treatments (SI Fig. 7 panel D, $P < 0.001$). Within enclosures, $T_{fluc/mean}$ of the water was higher in bare soil compared to *C. helmsii* dominated treatments (SI Fig. 7 panel D, $P < 0.001$). *Crassula*-dominated locations outside enclosures showed an intermediate $T_{fluc/mean}$, but were not significantly different from bare soil enclosures.

Discussion

The presence of *C. helmsii* resulted in multiple effects on the reproduction of *E. calamita*. Egg strings were typically found to be deposited predominantly on locations with a lower cover of *C. helmsii*. The average percentage of hatched eggs was also found to be lower in enclosures with a dominant soil cover of *C. helmsii*. Total length of tadpoles was however significantly higher, and growth and development to adult stages was faster in the enclosures with *C. helmsii* dominated soil.

C. helmsii significantly affects abiotic parameters of the water column where thick and dense vegetation

covers the littoral zone (SI Table 6). The pH was significantly lower in *C. helmsii* dominated enclosures in comparison to bare soil enclosures. At the end of the field measurements, ammonium and nitrate concentrations were significantly lower in enclosures containing *C. helmsii*. The enclosures with *C. helmsii* dominated soil retained more water and had lower water temperatures in comparison to the enclosures with bare soil. This resulted in lower evaporation rates and thus lower total evaporation. The higher volume of water loss in enclosures without *C. helmsii* dominance resulted in a significant increase of ion concentrations (i.e. ammonium, nitrate) at the end of the experiment. Daily temperature fluctuations were indeed significantly smaller in *C. helmsii* dominated enclosures compared to the bare soil enclosures resulting in a lower minimum day time temperature in *C. helmsii* dominated enclosures.

Effects of *C. helmsii* on spawning and hatching

Our study found a negative effect of *C. helmsii* dominance on spawning site selection and hatching success. Spawning did not take place at random. Sites overgrown by the invasive plant species were clearly avoided for spawning. Instead, *E. calamita* selected sites with sparsely vegetated or bare soils. The species' preference of spawning in bare locations is confirmed by several other studies in Europe (e.g., Lopéz-Jurado, 1983; Sinsch, 1998; Pujol-Buxó et al., 2019; Smith, 2019) and specifically pointed out for the Netherlands (Bij12, 2017). It remains unclear whether *E. calamita* stops spawning when the waterbody is completely dominated by the invasive plant species, especially because several authors report of spawning in densely vegetated pools (Reyne et al., 2021, personal observations by W. de Vries in Denmark, M. van de Loo in Spain, France and Poland, J. van der Loop in the Netherlands, and R. Rannap in Estonia and Denmark). However, our results also show that hatching success of eggs under *C. helmsii* dominance is lower. Our observed dampening of microclimatic daily temperature fluctuations caused by *C. helmsii* inside as well as outside enclosures is a plausible explanation for this phenomenon. High irradiation and resulting higher daytime water temperatures are known to be beneficial for egg development (Banks & Beebee et al., 1988; Pujol-Buxó et al., 2019) and survival (Sanuy et al., 2008). Therefore, we

conclude that high *C. helmsii* cover can pose a threat to the survival of *E. calamita* populations.

Effects of *C. helmsii* on larval growth

Contrastingly, larval growth and onset of metamorphosis were considerably faster under *C. helmsii* dominance than on bare soils. It is unclear why tadpoles accelerated their growth under *C. helmsii* dominance. One possible explanation is that the higher initial density of tadpoles in bare soil enclosures (due to higher hatching success) resulted in higher competition, ultimately affecting growth rate (e.g., Heusser, 1972; Tejedo & Reques, 1994). However, incorporating the initial number of tadpoles present in the GAM formulations did not result in a significant improvement in model fit, suggesting that density effects on larval growth were small in our experiment. This is in agreement with Beebee (1979) and Brady & Griffiths (2000), who concluded that *E. calamita* shows low sensitivity to intraspecific competition. Additionally, in natural situations higher densities of larvae are no exception (e.g., Sinsch, 1998). We also hypothesized that high nutrient uptake by the invasive plant would reduce the growth of algae, thus reducing availability of the most important food source for tadpoles. This was clearly not the case since dissolved nutrients were lower in enclosures dominated by *C. helmsii* but this did not result in reduced growth of tadpoles. An alternative explanation is that the structure of *C. helmsii* leaves creates an enlarged substrate for algae and thereby increases the availability of algal food. Since we did not measure algal biomass in the water column nor the biomass of algae present on plant tissue, this hypothesis requires further investigation.

Another explanation of changes in larval growth in *C. helmsii* dominated enclosures concerns multiple significant effects of *C. helmsii* on physical and chemical properties of the water. Evaporation and drying up of the lake had a strong concentrating effect on various dissolved chemicals. The effect of pH during the last two measurements might have resulted from decomposition of organic matter at higher day temperatures. However, this occurred at the end of the experiment, well after tadpoles had been able to grow into the metamorph stage. Low pH and/or high concentration of ammonium and aluminium can have a lethal effect on *E. calamita* tadpoles (Leuven et al., 1986; Sinsch, 1998; Fedorenkova et al., 2012). However, all concentrations of measured water chemistry parameters were within ranges of optimal breeding sites of *E. calamita* in Northern Europe (SI Table 11). When comparing bare soil and *C. helmsii* enclosures, *C. helmsii* did significantly affect several water chemistry parameters. *C. helmsii* reduced pH slightly (from 6.3 to 6.0), probably because of residual products originating from storage and breakdown of malic acid during CAM-photosynthesis of *C. helmsii*. This is beneficial for this plant species as CO_2 concentrations are higher at a lower pH, and the plant is unable to use HCO_3^- for photosynthesis (Newman & Raven 1995). However, pH did not decrease to values that could have significantly affected larval fitness. *E. calamita* is able to survive in waters with a pH ranging from 4.3 to 8.6 (Sinsch, 1998), and the lethal pH for tadpoles is below 4.3 (Leuven et al., 1986; Federenkova et al., 2012).

Concentrations of ammonium were lower in enclosures dominated by *C. helmsii*. Inorganic nitrogen was most probably reduced due to uptake by plants for growth as well. Effects on *E. calamita* are not expected as this species is known to tolerate the measured maximum NH_4^+ concentrations. Measured pH values were circumneutral and could therefore not add to ammonium toxicity. Other studies also failed in establishing a relationship between ammonium and growth or development of tadpoles (Bregulla, 1986; Dannefelser & Sinsch 1993; Federenkova et al., 2012). However, the production of algae, which depends on inorganic nitrogen availability (among other parameters), could be altered resulting in a different composition of algae species and biomass of algae available for tadpoles (e.g., Smith, 1983a, b; Piorreck et al., 1984).

During the experiment, water in the enclosures started to evaporate resulting in higher concentrations of ions, as is apparent from the increase in inert sodium and chloride. The increase in concentrations of sodium and chloride are not expected to negatively affect tadpoles of *E. calamita* as they successfully survive in habitats with broader ranges of these ions (Sinsch, 1998).

Effects of differences in water temperature on larval growth (Golay, 1996; Sinsch, 1998) could also not provide a satisfactory explanation for the increased larval growth in *C. helmsii* dominated enclosures. Scaled fluctuation was lower in *C. helmsii* dominated enclosures compared to bare soil enclosures.

For eggs, this microclimatic dampening effect of *C. helmsii* is the most plausible explanation for reduced egg hatching success. For tadpoles, this is apparently less important. High temperature extremes in bare soil treatment may have resulted in increased temperature stress for tadpoles. However, mean temperature maxima as well as daily fluctuations were markedly higher outside the enclosures (possibly due to shading effects of wall and the covering mesh of the enclosures), undermining this explanation. Other studies on *E. calamita* have shown that higher water temperatures result in accelerated growth due to accelerated metabolism (Smith-Gill et al., 1979; Sanuy et al., 2008,). However, these authors only refer to the average temperature over 24 h, and diurnal temperature fluctuations are not included. In our experiment, average water temperature in *C. helmsii* dominated enclosures did not differ from the bare soil treatment, and was comparable to those encountered outside the enclosures. Temperature maxima in bare soil enclosures were higher and minima were lower in *C. helmsii* dominated enclosures. During the experiment, tadpoles in *C. helmsii* dominated enclosures were observed to mainly graze on top of the vegetation, directly below the water surface, probably enabling the tadpoles to increase their exposure to solar irradiation. Tadpoles in bare soil enclosures were observed to mainly graze on the bottom of the enclosure, and not on the water surface of the enclosure. Thus, tadpoles were able to select for their preferred temperature environment in both treatments, resulting in two distinct behavioural choices. Such sun exposed enclosures with warmer water, will probably optimize temperature for grazing and growth of larvae.

The lower water level observed in enclosures containing bare soils might explain the smaller size attained by tadpoles in these enclosures. Water levels differed between treatments because enclosures placed on soils dominated by *C. helmsii* were on average located deeper in the waterbody. The combined effect of changes in water level and vegetation may have caused the difference in larval development speed. Water levels and the speed of drying-out influenced larval development (e.g. Tejedo & Reques, 1994; Brady & Griffiths, 2000). Tadpoles of *E. calamita* accelerate their development during desiccation of a water body, resulting in smaller larvae entering metamorphosis. The drying out coincided with a shorter body length of tadpoles reaching metamorphosis, but did not lead to faster larval development in the bare soil treatment.

Thus, we conclude that larval growth was significantly higher in *C. helmsii* dominated enclosures than on bare soil, but that differences in measured abiotic conditions cannot explain this phenomenon. We hypothesize that algal food could be higher in *C. helmsii* treatments, as the leaves and stems of the plant increase the surface area for algae growth and thus availability of algae. To what extent this benefits the tadpoles warrants further research.

Limitations of the study

The experiment did not include a control with enclosures dominated by native plant species instead of an alien invader as native vegetation was absent at our study site. One major consequence of this lack of a positive control is that we cannot prove that similar effects on *E. calamita* are found when ponds are increasingly populated by native vegetation, hence reducing bare ground and increasing vegetation cover. Since the location was managed to maintain a bare soil environment, effects of high native vegetation cover could not been assessed in past nor future. The rapid growth of invasive *C. helmsii* renders this management goal increasingly difficult to reach. However, since Reyne et al. (2021) found that spawning of *E. calamita* was associated with a high percentage cover of aquatic vegetation in lakes, but with short terrestrial vegetation in the surrounding vicinity, it may be possible that egg deposition and successful reproduction are not hindered by an increase of vegetation.

Unfortunately, we were not able to maintain equal water levels between different treatment groups over the course of the experiment, as the bare soil treatments dried out faster because of shallower placement. This was an unforeseen uncontrolled small-scaled effect of the locations as temperatures and water evaporation were higher than expected for this time of year. Consequence is that the measured differences were due to placement rather than treatment.

Conducting a field study can always yield unexpected variables. However, we do not feel that performing this experiment in a controlled laboratory environment is justified due to the declining population in this 'De Gijzenrooise Zegge' of this protected amphibian, and various infectious disease risks associated with transport of egg strings, tadpoles and

juveniles. By conducting the experiment at the field location, the population of *E. calamita* experienced as little disturbance as possible. In addition, variables that are natural but cannot be simulated in the laboratory are available thanks to implementation in the field. Conducting the study in the field may more accurately reflect biological reality, due to the incorporation of these additional variables. However, we think a laboratory version to have other, comparable consequences.

Demographic implications

C. helmsii alters demographic parameters of *E. calamita* populations, since it significantly reduces egg spawning and hatching success where it is dominant, i.e. with a dense vegetation cover of 95–100%. Number of egg strings is positively correlated with number of tadpoles (Reyne et al., 2021), and lower egg production results in a lower reproduction success. Although counting the number of egg strings cannot be used to directly calculate population size (Schmidt, 2004, 2005), it serves as a time efficient indication of the relative change in the number of females spawning over time. It remains unclear whether nature areas become completely unsuitable for reproduction of *E. calamita* when *C. helmsii* dominates an area, causing this species to become locally extinct. In cases of low reproduction rate and deteriorated habitat quality, local extinction of populations of *E. calamita* can quickly occur (Zahn et al., 2020). However, growth is accelerated in our enclosures under *C. helmsii* dominance. This can be beneficial for *E. calamita* as larger tadpoles at metamorphosis, with wider heads and thicker hind legs, may exhibit increased feeding, fleeing and dispersion capability, resulting in increased survival as toads (Tejedo et al., 2010). Additionally, a larger size at metamorphosis may increase reproductive success as larger males are more attractive and larger females can produce bigger clutch and eggs (Tejedo 1992a,b). Our experiments could however also simply indicate that tadpoles prefer aquatic vegetated patches over bare soil. In this case, heterogenic shallow water habitats containing bare soil areas for egg development as well as vegetated areas for larval development are probably most suitable as breeding sites for *E. calamita,* similar to findings of Reyne et al., 2021. Without control measures however, invasive *C. helmsii* completely overgrows these habitats,

and patches of bare soil, and the presence of standing water, will quickly disappear (SI Fig. 8). Additionally, in North-western Europe, shallow lakes similar to our study site are normally characterized by native plant species of the *Littorellion uniflorae* plant association. The growth forms of these species (i.e. *Littorella uniflora* (L.) Rusby, *Pilularia globulifera* L. and *Baldellia ranunculoides* subsp. *repens* (Lam.) Á.Löve & D.Löve) remarkably differ from that of *C. helmsii* (a low-growing vegetation with isoetid habit that grows slowly versus faster biomass production and higher plant growth of the invader) (Den Hartog & Segal, 1964; Bloemendaal & Roelofs, 1988; Dawson & Warman; 1987). *C. helmsii* causes different soil conditions, i.e. because of altered light penetration, and therefore will differentially affect spawning behaviour of *E. calamita.*

All things considered, this study still leaves considerable uncertainty about the demographic implications for *E. calamita* when *C. helmsii* is present. However, the negative effect on spawning and hatching success, in combination with the high impact of *C. helmsii* infestation on vegetation structures (e.g., Dawson & Warman, 1987; Leach and Dawson, 1999; Van Kleef et al., 2017; Smith & Buckley, 2020) justifies erring on the side of caution and we recommend to implement management of *C. helmsii* in breeding habitats of *E. calamita.* Future research and demographic modelling of *E. calamita* reproduction in breeding sites recently infected by *C. helmsii* will assist in elucidating the ultimate effects on population viability of *E. calamita*. In such research, special attention should be paid to further unravel the causal mechanisms determining the speed of larval growth under *C. helmsii* dominance, not only relating to availability of bare soil but also to varying cover of native vegetation. In addition, it is recommended to determine the effects of *C. helmsii* on other amphibian species.

Implications for invasive species management for biological conservation

Our research clearly shows that *C. helmsii* negatively affects spawning and hatching success. On the other hand, a faster growth rate of tadpoles is observed at soils dominated by *C. helmsii*. This indicates that the population is possibly not negatively affected by the presence of both bare soils and *C. helmsii* dominated

soils. However, in north-western Europe, suitable habitats of both species are almost identical which poses a threat to *E. calamita* when massive overgrowth of its breeding sites by *C. helmsii* occurs. The plant species has a tendency to completely suffocate a waterbody by its unlimited growth and can make waterbodies dry out within a few years making waterbodies unsuitable for *E. calamita* without management (e.g. Smith & Buckley 2020; Van der Loop et al., 2019, 2020). This is expected in the study area as the plant species has only recently spread in the area and expansion of *C. helmsii* is still ongoing.

An increased effort will be required to protect this Annex IV Habitat Directive species and its habitats as populations of *E. calamita* are declining due to a shortage of suitable breeding waters and insufficient nature management, among other causes (Husté et al., 2006; Stevens & Baguette, 2008; Goverse, 2009; Beebee et al., 2012). *C. helmsii* will probably be contributing to this decline given its rapid range expansion and dominance of many freshwater pioneer habitats. Even though eradication of *C. helmsii* is difficult and very costly (Van der Loop et al., 2018, 2022), measures to prevent spread and population control of this invasive alien plant species need to be taken into account in management of breeding sites of *E. calamita*.

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Declarations

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