

Different behavioral profiles between invasive and native nudibranchs: means for invasion success?

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Abstract

Behavior is predicted to be a primary determinant of the success of the invasion process during the early phases of colonization. Comparing invaders with sympatric native species may provide a good approach to unravel behavioral traits involved in an invasion process. In this study, we carried out an experimental simulation of the introduction and the acclimatization phase into a new environment and assessed the expression of activity, alertness, and habituation in an invasive Mediterranean population of the South African nudibranch *Godiva quadricolor* comparing its profiles with those of the sympatric Mediterranean native nudibranchs *Cratena peregrina* and *Caloria quatrefagesi*. Individuals of these 3 species were subjected to 3 behavioral tests: spontaneous activity, carried out in the introduction phase (immediately after sampling) and after a week of acclimatization; alert test, in which a potential threat was simulated by means of a tactile stimulus, and habituation test, in which the same alert test stimulus was repeated 5 times at 30-min intervals. The invasive *G. quadricolor* showed higher levels of exploration activity, thigmotaxis, alertness, and sensitization than the native species. These behavioral traits may represent pivotal drivers of the ongoing invasion process.

Key words: biological invasion, invasive phenotype, invertebrate cognition, molluscs, personality.

Biological invasions have surged in the last century, emerging as significant challenges in ecology (Essl et al. 2011; Lewis et al. 2016; Roy et al. 2023) and primary drivers of biodiversity loss and habitat disruption at local and regional scales (Gilbert and Levine 2013; Fritts and Rodda 1998; Kaufman 1992; Clavero and García-Berthou 2005; Davis 2003; Gurevitch and Padilla 2004; Sax and Gaines 2008). Behavioral and personality traits favoring the dispersal and establishment of new populations are predicted to exert a pivotal contribution to the success (Carere and Gherardi 2012; Chapple et al. 2012) and impact (Jette et al. 2014) of biological invasions. Underscoring the importance of behavioral mechanisms enabling certain species, although not others, from successfully spreading outside their natural geographic ranges thus represents a compelling field of research (Cucherousset and Olden 2011; Essl et al. 2011; Lewis et al. 2016; Blight et al. 2018; Ruland and Jeschke 2020). The success of a population in adapting to changes associated with a completely novel landscape depends on its ability to adapt quickly (Tuomainen and Candolin 2011; Tabosky 2017). Although selection may favor some behavioral phenotypes over others, the degree to which an organism can plastically amend behavior, is also crucial in determining the outcome of an invasion (Chapple et al. 2012, 2022; Griffin et al. 2016; Cordeschi et al. 2022).

Individuals carrying correlated suites of traits (i.e., behavioral syndromes, Sih et al. 2012) may have an advantage during the introduction into new environments. For

example, aggressive individuals are generally bold and exploratory (Rehage and Sih 2004; Cote et al. 2010) and this may enhance their chance to interact with transport vectors (I° invasion stage), establish a non-native population through the exploitation of local resources (II° stage) and spread to new habitats by means of competitive advantages over native species (III° stage) (Blackburn et al. 2011). Theoretical models, encompassing concepts like spatial selection and spatial sorting, forecast traits enhancing dispersal abilities to be distributed non-randomly in space. The genetic imprints of these traits might endure along the different colonization phases of the new environment (Phillips et al. 2010; Cobben et al. 2015; Canestrelli et al. 2016). Novel invaders indeed may represent a non-random subset of a source population with a strong expression of personality traits associated with dispersal abilities such as exploration and/or aggression (e.g., Shine et al. 2011; Canestrelli et al. 2016). For example, in avian species, cognitive abilities and exploration tendencies were enhanced in those species that successfully established an invasive population, compared with those that did not, as well as in individuals on the invasion front compared with individuals from long-established populations (Griffin et al. 2016). Furthermore, studies on invasive lizard species have provided in-depth insights, revealing that heightened exploration behavior and boldness are correlated with the successful establishment and expansion of new populations (Chapple et al. 2012).

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Comparative studies between invaders and sympatric, closely related, native species provide a good opportunity to detect functional behavioral traits and selective forces at play behind the invasion process, like dispersal (Holway and Suarez 1999; Rehage and Sih 2004; Damas-Moreira et al. 2019), feeding (Rehage et al. 2005) and bold behaviors (Pârvulescu et al. 2021). The number of empirical studies and competing hypotheses about biological invasions has increased over time (Richardson and Pysek 2008; Enders et al. 2020), yet most behavioral studies have focused on terrestrial species, despite, for example, marine invertebrates representing a significant portion of global biodiversity (Brembs 2013). These studies often include comparative analyses with native species (Damas-Moreira et al. 2019, 2020), enriching our comprehension of how specific behavioral traits contribute to the invasive success of certain species.

The South African nudibranch *Godiva quadricolor* is a species native to the Indian Ocean that spread to different tropical and temperate areas in the last century (Cervera et al. 2010). *G. quadricolor* was likely unintentionally introduced in the Mediterranean Sea as stowaways by the commercial mollusk trade and ballast water (Cattaneo-Vietti et al. 1990; Villani and Martinez 1993; Willan 2004; Cervera et al. 2010). All conspicuous populations have been reported only from brackish waters and coastal lagoons used for aquaculture purposes (Macali et al. 2013; Furfaro et al. 2018; Kučić and Lanča 2018), which may thus be considered the expanding edge of the invasion front. Among the most common native nudibranchs in coastal lagoons and marinas where the invasive populations have established (Cattaneo-Vietti and Chemello 1991; Macali et al. 2013; Kučić and Lanča 2018), the Mediterranean facelinid nudibranchs *Cratena peregrina* (Gmelin, 1791) and *Caloria quatrefagesi* (Vayssière, 1888) are frequently found in sympatry with *G. quadricolor* (e.g., Macali et al. 2013; Betti et al. 2017). All 3 species are diurnal and conspicuously stand in the open, forming dense populations with high dispersal potential. Given that both invasive and native species populations are established in homogenous artificial landscapes, it can be assumed that they have access to similar microhabitats. However, to the best of our knowledge, *C. peregrina* and *C. quatrefagesi* have not been successfully introduced outside of their native range.

In this study, we experimentally simulated the introduction and acclimatization of the aforementioned species to a new environment and repeatedly quantified spontaneous locomotory activity, alertness, and habituation on the hypothesis that such behavioral traits are involved in the initial phases of the invasion process. We therefore expected the invasive species to display higher activity levels and quicker responses to environmental stimuli when faced with a novel environment than the native species.

Materials and Methods

Sampling, housing, and experimental setup

Individuals were sampled in Mediterranean coastal lagoon environments between April 2019 and July 2020. Considering the tendency of the invasive *G. quadricolor* to prey on other nudibranchs, such as the two sympatric species investigated in our study, and to prevent any effect of this predatory pressure on the behavior of the 2 native species (Abrams 2000), we selected Mediterranean species sampling sites in ecologically suitable areas in which the presence of the invasive species was

not ascertained. To do so, a total of 34 individuals for each of the native *C. quatrefagesi* and *C. peregrina* were collected from the Nassa Channel of Orbetello Lake (Italy, Tuscany; 42°25'59"N-11°09'30"E; 1m depth on rocky substrate) and 32 specimens of the invasive species from the Roman Channel of Sabaudia Lake (Italy, Latium; 41°14'50"N-13°02'16"E; 1–2 m depths on rocky-mud flats). For the invasive species, specimens were collected in 2 different samplings due to logistic reasons.

All individuals were placed in separate plastic bottles filled with 0.5 L of marine water and transferred within 2 h to the CISMAR (Marine Ichthyogenic Experimental Center, University of Tuscia) facilities. Sampled specimens were photographed inside a Petri dish placed on graph paper and measured in length with the help of *ImageJ* software v 1.53 (Abràmoff et al. 2004) and randomly maintained in floating arenas placed in a 1000 L fiberglass tank supplied with aerated running seawater with salinity and temperature set at environmental sampling conditions ($S = 35\%$, $T = 21.5\text{ }^{\circ}\text{C}$; see Figure 1A for details). They were fed *ad libitum* daily according to specific diets: *C. quatrefagesi* and *C. peregrina* were fed with *Eudendrium* sp. Like most predatory opisthobranchs, the invasive species *G. quadricolor* is considered a generalist species and euriphagy is well-known also in specialized carnivore nudibranchs (Megina et al. 2007); accordingly, animals were fed daily *ad libitum* with *Artemia franciscana* and other nudibranch species. To maintain consistency among the treatment groups and minimize the impact of external variables, animals that did not feed daily were excluded from the tests.

Behavioral assessment

Spontaneous activity

We measured spontaneous locomotory activity by video recording immediately after the disposal in the floating arenas (*C. peregrina* and *C. quatrefagesi* $\varnothing = 57\text{ mm}$; *G. quadricolor* $\varnothing = 200\text{ mm}$), about 3 h after the sampling, which simulates a sudden introduction into a new environment. The same measurements were taken 1 week later, aimed to simulate an acclimatization period. We considered 2 replicas of the test only for the acclimatization phase (7th and 8th day after the sampling) excluding the introduction trial to prevent any biases in the test (Figure 1B). In the context of the statistical analyses conducted on these data, we considered the mean values derived from the 2 replicates for each individual. Given the differences in body length between species, we set up arenas proportionally to the average body size of the species, and grouped them together, according to the different magnification settings required ($n = 16$ for *G. quadricolor* and $n = 9$ for *C. peregrina* and *C. quatrefagesi*). Videos were taken using a GoPro 5 set on time-lapse mode with a frame rate of 1f/5 s for 120 min, with a resolution of 1920 × 1080. The camera was mounted on a flexible “gooseneck”-style mounting arm for easy positioning in the center of the arena block (Figure 1A).

Processing tracks to measure locomotor activity were performed with Tracker Video Analysis and Modeling Tools v5.1.5 (Open-Source Physics; <https://physlets.org/tracker/>). Each time-lapse video was analyzed with a frame-by-frame method. The position of body control points was fixed on the upper part of the head due to its good visibility in each frame and replicability of selection. Before measurements, calibration was set according to the arena width. Motion analysis allowed the determination of *Distance*, *Average Speed*, and

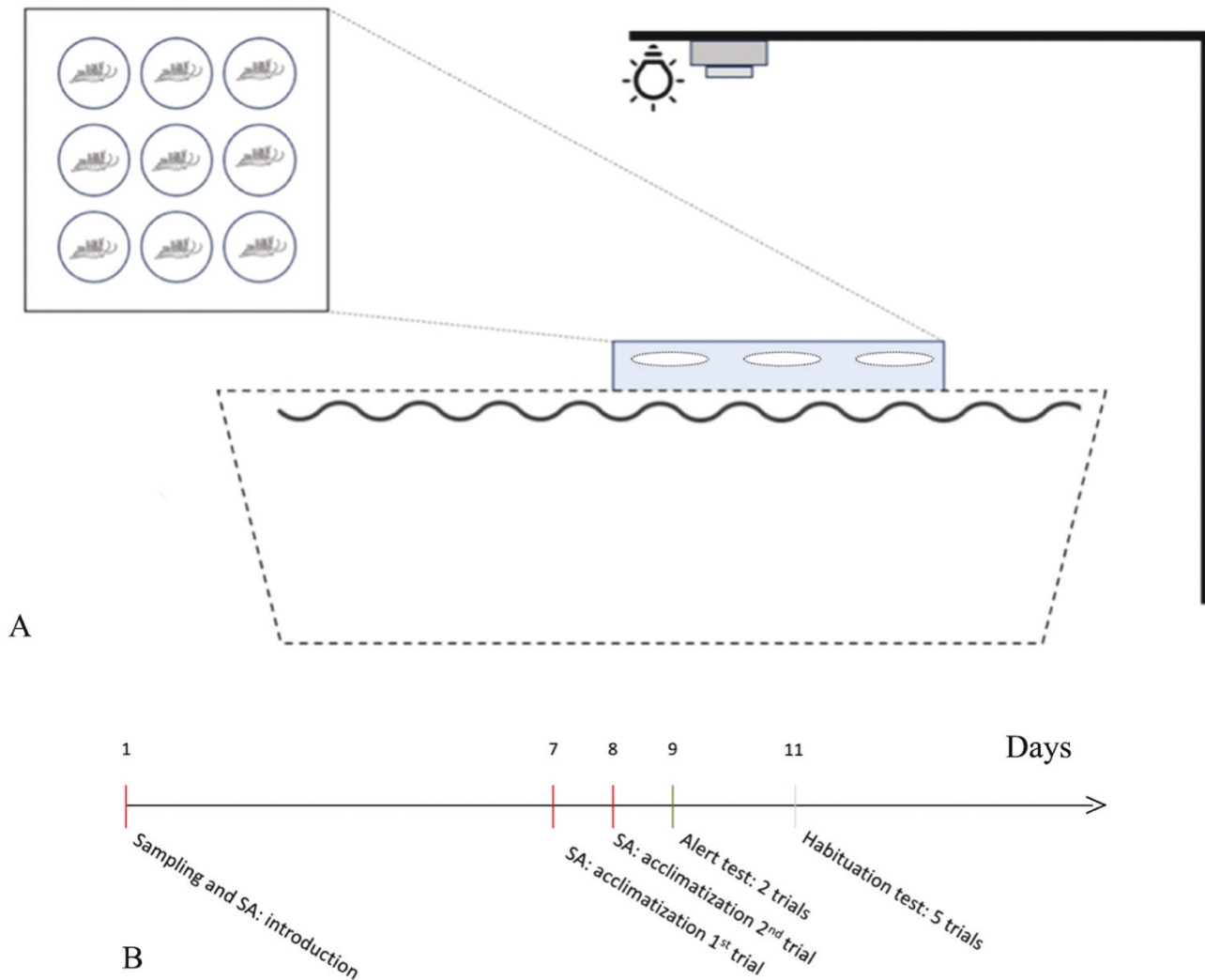


Figure 1. Experimental setup: (A) spontaneous activity floating arena; (B) timeline of the behavioral tests (red: spontaneous activity (SA)); green: alert test; light blue: habituation test.

Average Acceleration (hereafter defined as *Distance*, *Av.Sp.* and *Av. Acc.*). We used the path track to quantify the thigmotaxis (hereafter defined as *Thigmo*), calculated as the percentage of path (as number of pixels) in the external ring (15 mm) of the arena over the whole path tracked.

Cerata extension response: alert and habituation test

Nudibranchs use cerata as a defensive tool against predators (Faulkner 1992; Gavagnin et al. 1994; Aguado and Marin 2007), displaying their active movement and extension in response to different stimuli under control of peripheral nerves and muscle contraction (Bickell-Page 1989). The individual cerata response to a tactile stimulus and the escape reaction (Video 1) were video recorded for 5 min right after the stimulus. The time elapsed between the extension of cerata as a reaction to the stimulus and their complete relaxation was measured as well as the occurrence of the escape response; we also considered the “*Escape Index*” (index = 1 when animals escape; index = 0 when they did not escape) in all trials for both the alert and the habituation tests (Table 1). In the tests, animals were individually transferred into smaller arenas, with 30 min of acclimatization before each test. We used a soft plastic string (10 × 3 × 0.1 mm) to gently

apply the tactile stimulus in the median dorsal part of the animal, enough to induce cerata extension. We used this procedure in 2 different tests carried out on 2 consecutive days differing in the time intervals between stimuli: in the *alert test*, 2 trials with an interval of 12 h were conducted (Th0, Th12), whereas in the *habituation test*, 5 trials with an inter-trial interval of 30 min were conducted (Tm0, Tm30, Tm60, Tm90, and Tm120) (Figure 1B).

Statistical analysis

Statistical analyses were performed with RStudio, (R version 4.3.1 (2023-06-16)—“Beagle Scouts” and Rstudio version 2023.06.2 + 561 RStudio: Integrated Development for R. RStudio, Inc., Boston, MA, URL <http://www.rstudio.com>). Descriptive statistics are reported as mean ± standard deviation and the significant threshold of the statistical test was set at $P = 0.05$. If measured variables did not meet the assumption of normality and/or homoscedasticity among groups, we log-transformed or root-squared transformed them to increase model fitting. The native species were significantly smaller in length than *G. quadricolor* (one-way Anova: $F_{2,95} = 252.54$, $P < 0.001$; paired Bonferroni-post hoc *t*-test: *C. peregrina* and *G. quadricolor* $P < 0.001$; *C. quatrefagesi* and

Table 1. Summary of the behavioral tests and parameters measured in the invasive (*G. quadricolor*) and the native (*C. peregrina*, *C. quatrefagesi*) species. #: sample size.

Behaviour	Time	# <i>C. peregrina</i>	# <i>C. quatrefagesi</i>	# <i>G. quadricolor</i>	Trait
Activity					Distance (m) Average speed (m/s) Average acceleration (m/s ²)
<i>Introduction</i> (1 trial)	2 h	30	20	21	
<i>Acclimatization</i> (2 trials)					Thigmotaxis (% of path on external ring over the whole path tracked)
Cerata extension					Time(s) of cerata extension after stimulus
<i>Response</i>	5 min	29	15	13	Occurrence of escape response. Escape index (see text)
<i>Alert test</i> (2 trials)					Time(s) of cerata extension after stimulus Occurrence of escape response.
Cerata extension					Δt : difference in time (s) between the first and the last trial in the habituation test
<i>Response</i>	5 min	22	15	10	Δe : difference in the frequency of escape behavior (e) between the first and last trial in the habituation test (e = escaping animals/N*100) Escape index (see text)
<i>Habituation test</i> (5 trials)					

G. quadricolor $P < 0.001$), although the two native species did not differ ($P > 0.05$). All analyses were therefore carried out considering size as a covariate in model fitting.

Spontaneous activity

Due to logistic constraints, the invasive species was collected in 2 distinct samplings. These separate collections were treated as independent batches for the spontaneous activity test (introduction and acclimatization). In contrast, for the native species, a single batch was used for both experimental phases, as the animals were collected simultaneously. Accordingly, we performed the 2 independent analyses for both experimental phases with a simple linear model (“lm” function from “stats” Rpackage) fitted with each dependent variable (i.e., *Distance*, *Av. Sp.*, *Av. Acc.* and *Thigmotaxis*), and “Species” as main factor, considering each phase separately. We also explored the interaction between “Species” and “Size.” When the analysis revealed a significant effect of “Species” term and the interaction included “Species,” Bonferroni and Tukey-post hoc pairwise comparison on the mean values or on the slopes of interested dependent variables were performed for evaluating differences among species, specifically: we performed “emmeans_test” function from “rstatix” R package (Kassambara 2023) for considering the confounding effect of “Size,” whereas we used the “emtrends” function from “emmeans” R package (Lenth 2024) with “Size” as covariate when the interaction was significant.

Alert and habituation test

In the alert test, we used a linear mixed-effects model (LMM, function “lmer” from “lme4” Rpackage; Bates et al. 2015) for repeated measures fitted with individual *ID* as a random effect, and “Species” and “Trial” (coded as “Th0” and “Th12”) as main factor to account for significant effect on cerata response, also including the interaction between these terms. “Escape index,” observed as a response to the

stimulation during the alert test, calculated as the difference between the escape response in “Th12” and “Th0” was coded as 0 = no escape response, 1 = escape response, was analyzed with a binomial simple linear mixed model (function “glmer” from “lme4” Rpackage, family distribution “Binomial”) fitted with “Species” and “Trial” as main factor as well as their interaction for comparing the probability to escape across species between two trials. The “Size” (log-transformed) was considered as a covariate in the model.

In the habituation test, we employed a similar model approach to analyze cerata response fitted with “Trial” coded as “Tm0,” “Tm30,” “Tm60,” “Tm90” and “Tm120” and “Species” as main factor.

Significant effects on dependent variables were assessed via Satterthwaite’s degrees of freedom method (“Anova” function from the ‘lmerTest’ R package; Kuznetsova et al. 2017). When finding a significant effect of main factor “Species” or interaction included such factors, we further investigated differences between species by performing pairwise post-hoc analysis adjusted for multiple comparison as previously described (“emmeans_test” function from “rstatix” package). The “Escape Index” was assessed via binomial general linear mixed model fitted as previously described in the alert test. For each mixed-effect models used for investigating the time response of alertness and habituation, we calculated the adjusted intraclass coefficient criterion (ICC) for evaluating the proportion of the variance explained by the grouping structure in our sample, that is, the random term “Individual.” The ICC provides a meaningful individual variation in behavioral plasticity when the total variance of the model is moderately explained by among-individual clustered variances (Bell 2009; Dochtermann 2023). Given that the significant main-effect (i.e., “Species” term) and individual variation (random factor) in explaining responses observed in both the alertness and habituation, we qualitatively explored phenotypic behavioral correlations emerging by covariance among behaviors

explained from among-individual differences. By following the approach of Houslay and Wilson (2017), we performed a bivariate mixed model using the R package ‘MCMCglmm’ (Hadfield 2010) separated for the three species. We fitted the multivariate model with time response of alertness and habituation as response variables (scaled as mean = 0, deviation standard 1 for model fitting), individual ‘Size’ as covariate and ‘Individual’ as random factor. We used an uninformative parameter-expanded prior for the random effects ($V = 1$, $\nu = 2$) and we ran the model for 42,000 MCMC iterations, with a burn-in of 20,000 and a thinning interval of 100. Based on the 4000 posterior samples, we calculated the mean for the pairwise correlations of the 2 behavioral traits by dividing the covariance between 2 behaviors by the product of the square roots of their variances. Estimated correlation coefficients and 95% confidence intervals were plotted to depict behavioral correlations among species.

Behavioral correlations among species

To understand phenotypic correlations involved in determining behavioral differences among the 2 native and the invasive species, we performed a correlation analysis followed by a principal component analysis (PCA) on those traits relevant for describing behavioral differences on exploration activity among species, that is, behavioral measurements observed in the introduction test, alert and habituation test (Budaev 2010; Stuber 2022). Due to the lack of significant differences in explaining escaping response of individuals among species, we decided to not further consider the escape index measured in the alert and habituation test. The new datasets (Table 2) resulted suitable for factor analysis (Kaiser–Meyer–Oklin value was 0.64; Bartlett’s test of sphericity was significant: $\chi^2_{15} = 135.096$, $P < 0.001$; Kaiser 1960; Dziuban 1974). The PCA allowed for an easier interpretation of data by summarizing several variables into a few components where the variance present in the data collected was maximized within each component and, potentially, explained phenotypic behavioral differences among species (Dingemanse et al. 2007; Wilson and Godin 2009; Smith and Blumstein 2010). The PCA was performed by using ‘prcomp’ function from ‘psych’ R package (Revelle 2023) after scaling and centering the correlation matrix of behavioral traits. The PCA found 2 main components that taken together explained 68.19% of the variance. The eigenvalue associated with the Second Component was higher than 1, however, close to the expected threshold

(Second Component eigenvalue: 1.348). The expected eigenvalue was calculated with the function ‘hornpa’ from ‘hornpa’ RPackage (Huang 2022) settled with sample size: 45, n . variables: 6, repetition: 1000: mean 1.254, 95th percentile 1.404.

For assessing phenotypic differences among species on the resulting 2 principal components, we performed 2-separated linear regression analysis (linear model) in which each component was fitted as the dependent variable, with ‘Species’ as the main factor and ‘Size’ as the covariate. Further, post hoc analyses were performed as previously described for assessing species differences in the introduction test.

Results

Spontaneous activity

The statistical analyses on the activity test revealed a general positive correlation between the size and all measured traits (*distance* $P < 0.001$; *Au. Sp.* $P < 0.001$; *Au. Acc.* $P < 0.001$; SM, Table 1), suggesting that individual size is an important state variable that is linked with the general propensity of individuals to explore.

Overall, we found a significant difference in locomotory activity among the species during the experiment (Figure 2). In the introduction phase, a significant difference among species was unveiled for each of the measured variables (*Distance* $P < 0.001$; *Au. Sp.* $P < 0.001$; *Au. Acc.* $P < 0.001$); *Thigmotaxis* $P < 0.01$) (SM, Table 1). Post hoc tests (SM, Table 2) revealed that *G. quadricolor* covered a larger distance (*C. peregrina*-*G. quadricolor* estimate: -3.32 and $P < 0.001$; *C. quatrefagesi*-*G. quadricolor* estimate: -6.36 ; $P < 0.001$) and displayed more thigmotaxis (*C. peregrina*-*G. quadricolor* estimate: -3.14 and $P < 0.010.007$; *C. quatrefagesi*-*G. quadricolor* estimate: -3.35 ; $P < 0.01$) compared with both native species. Considering the *Au. Sp.* (estimate: -2.82 ; $P < 0.001$) and *Au. Acc.* (estimate: -0.119 ; $P < 0.001$), *G. quadricolor* displayed greater activity compared with *C. quatrefagesi* but no difference emerged when compared with *C. peregrina*. Moreover, post hoc tests revealed a significant difference within native species, where *C. peregrina* covered a larger distance (estimate: -6.42 ; $P < 0.001$), displayed high *Au. Sp.* (estimate: -5.09 ; $P < 0.001$) and *Au. Acc.* (estimate: -0.04 ; $P < 0.001$) compared with *C. quatrefagesi*, whereas native species did not differ in their thigmotaxis behaviour. In the acclimatization phase: *G. quadricolor* also covered a larger distance

Table 2. Results of the principal component analysis (PCA) performed on the correlation matrix of the three species (sample size = 45; variables = 7). Scores values for each independent variable and the degree of prediction of each variable from others. Bold values indicate the variable that largely contributes to the specific component.

	Degree of prediction (KMO)	First component: “Exploratory activity”	Second component: “Learning ability”
Eigenvalue		2.74	1.345
Variance explained (%)		45.73	22.46
<i>Distance</i> (log-transformed)	0.68	0.57	-0.08
<i>Au.Sp.</i> (log-transformed)	0.74	0.58	0.03
<i>Au.Acc.</i> (root-squared-transformed)	0.68	0.55	0.14
<i>Thigmotaxis</i>	0.56	0.14	-0.68
Alertness	0.39	0.13	0.58
Habituation	0.19	0.08	-0.42

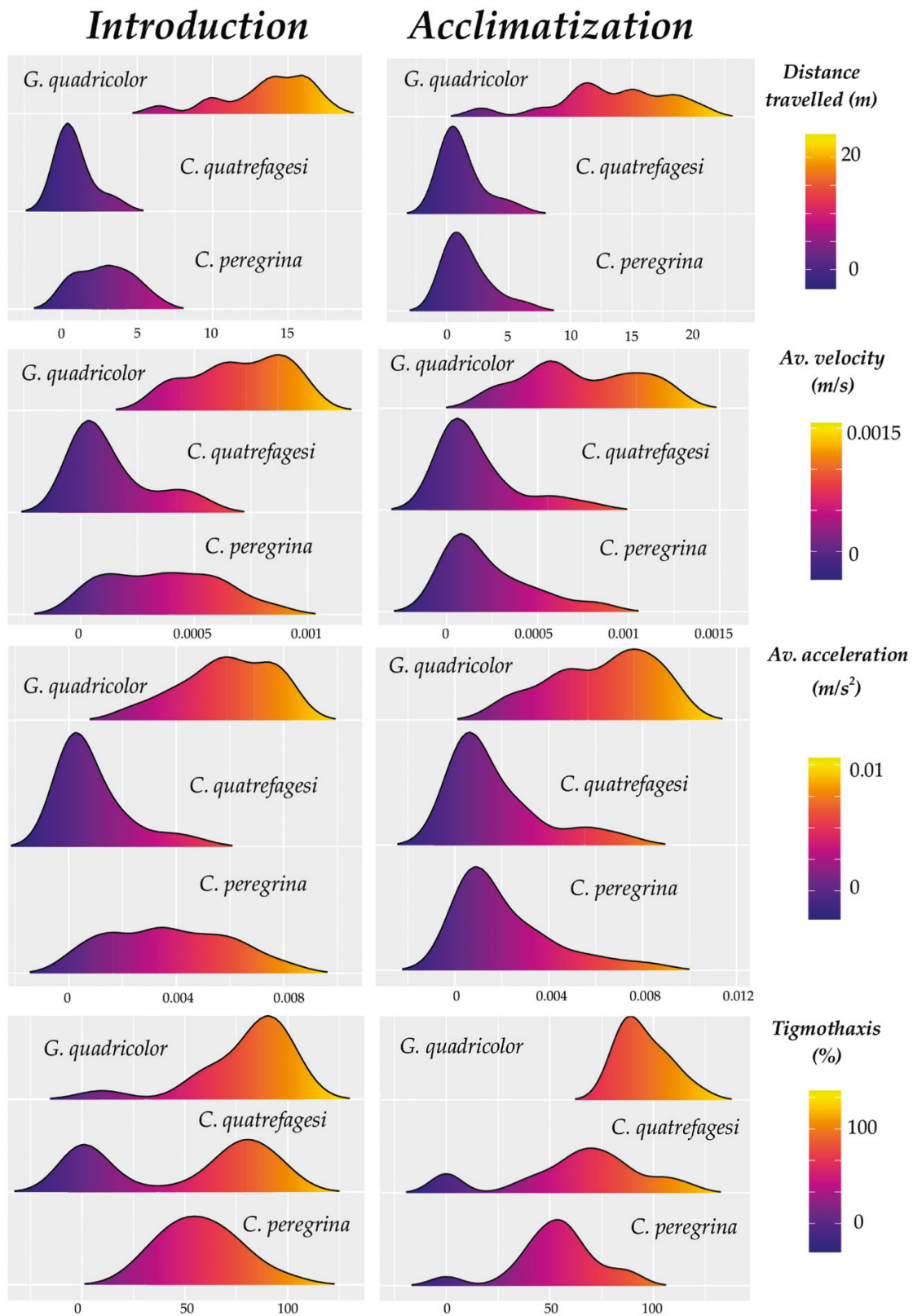


Figure 2. Kernel density plots displaying the estimated probability density of data of the four behavioral traits—distance, average speed, average acceleration, and Thigmothaxis—measured in the 3 species during both the introduction and acclimatization phases (non-normalized variables). For the acclimatization, we reported average values retrieved from the different trials. The plot unmistakably shows significant variability in the distributions of these measures. Notably, *G. quadricolor* consistently exhibits distinct peaks and shading patterns when compared with the native species.

(*C. peregrina*- *G. quadricolor* estimate: -2.84 and $P < 0.001$; *C. quatrefagesi*- *G. quadricolor* estimate: -3.74 ; $P < 0.001$) compared with both species, whereas *C. peregrina* showed

higher activity compared with *C. quatrefagesi* (estimate: -1.65 ; $P < 0.001$) (SM, Tables 1 and 2). No other behavioral traits were significantly different.

Cerata response: alert and habituation test

Differences in alertness were observed between native and alien species. Both *C. peregrina* and *C. quatrefagesi* showed longer times of alertness in the second trial (β estimated for *C. peregrina*: 0.74 ± 0.21 , $F_{1,25} = 12.482$, $P < 0.01$; β estimated for *C. quatrefagesi*: 0.42 ± 0.20 , $F_{1,14} = 4.293$, $P > 0.05$), whereas *G. quadricolor* quickly recovered from cerata extension (β estimated: -0.27 ± 0.11 , $F_{1,25} = 5.929$, $P < 0.05$), (Figure 3A). A significant difference in alertness response emerged between the species and the 2 tests (Alert test: main effect of “Species” $P < 0.001$; interaction “Species \times Trial” $P < 0.001$; Habituation test: main effect of “Species” $P < 0.001$; interaction “Species \times Trial” $P < 0.001$;) (SM, Table 3). In the Alert test, the post hoc analysis showed a significant difference in the alertness response only between *C. peregrina* and *G. quadricolor* in the second trial (Bonferroni-post hoc *C. peregrina* vs *G. quadricolor*: estimate: 3.41; $P < 0.05$), with the invasive species displaying a significant decrease of the alert response compared with the native. In the habituation test, both native species showed a slight reduction in the time of cerata extension (β estimated for *C. peregrina*: -0.16 ± 0.04 , $F_{1,87} = 21.680$, $P < 0.001$; β estimated for *C. quatrefagesi*: -0.18 ± 0.04 , $F_{1,59} = 19.410$, $P < 0.001$). On the contrary, *G. quadricolor* displayed an inverse profile, with a progressively increasing response (β estimated: 0.08 ± 0.05 , $F_{1,75} = 2.236$, $P > 0.05$). LMM confirmed significant differences between trials ($P < 0.01$) (SM, Table 3), also highlighted when considering the mean value of the ΔT , measured as the difference in cerata extension time between the first and the fifth trial as visually described in Figure 3B. Tukey-posthoc tests showed that both native species significantly reduced their response during the test compared with *G. quadricolor* (*C. peregrina* vs *G. quadricolor*: $P < 0.001$; *C. quatrefagesi* vs *G. quadricolor*: $P < 0.001$), whereas the native species did not differ ($P > 0.05$). The variance explained by the random term, i.e., “Individual,” in each model performed for the alert and habituation tests was higher than 40% (adjust ICC alert test:

0.556; adjust ICC habituation test: 0.443; likelihood ratio test of random-effects term in both model: $P < 0.001$), suggesting that the variance of behavioral response was moderately reliable and ascribed to individual differences.

The bivariate models did not find a significant between-individual covariation and correlation between the learning traits in the 3 species as reported in the large confidence interval which contained zero. *G. quadricolor* and *C. quatrefagesi* showed negative correlation values between the time response of alertness and habituation ($r = -0.04$ [CI = $-0.29; 0.16$] and -0.08 [CI = $-0.62; 0.43$], respectively) which was opposite in *C. peregrina* ($r = 0.03$ [CI = $-0.16; 0.244$]), but data did not statistically support the presence of behavioral correlations.

Escape response during the alert and habituation test

In Figure 4, Values of Δe , expressed as the difference in escape frequencies (escaping animals/ $N \times 100$) between the first and last trial in both the alert and habituation test, describe a clear different pattern in responsiveness between species.

In the alert test, however, the probability of escaping in response (Escape Index) to the stimulation did not differ among species (main factor species: $\chi^2_1 = 2.492$, $P = 0.114$) and the response did not change between trials (main factor trial: $\chi^2_1 = 4.197$, $P = 0.072$), nor the pattern of response across stimulation differed among species (species \times trial: $\chi^2_1 = 2.341$, $P = 0.310$).

The analysis performed on the escape index during the habituation test failed to detect an effect of “Species” ($\chi^2_1 = 3.146$, $P = 0.207$), the probability of escape response did not change across trials ($\chi^2_1 = 0.575$, $P = 0.448$), nor the interaction ($\chi^2_1 = 1.935$, $P = 0.380$).

Principal component analysis

Two dimensions explained 68.19% of the total variance; the first component contributed 45.73%, and the second component 22.46% (Table 2). The first component was mainly loaded by Size, Distance, Av. Sp., and Av. Acc., and these traits were

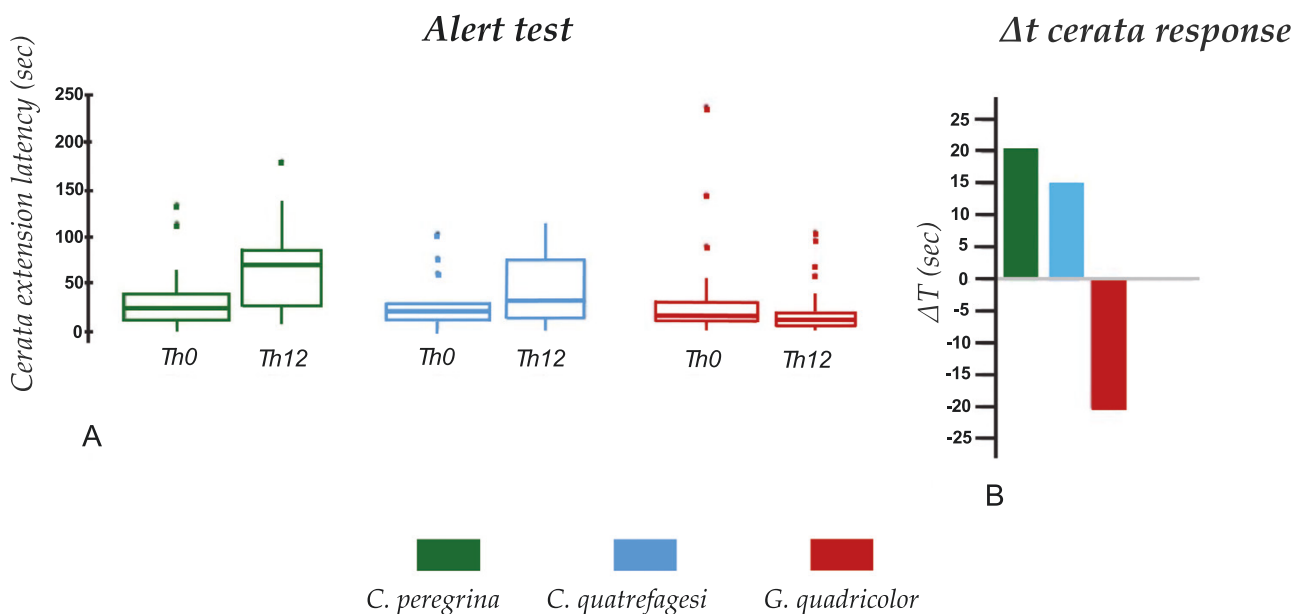


Figure 3. (A) Boxplot of cerata extension time in the Alert test. Scale axis in sec. Th0: first trial 30 min after the disposal in the floating arenas; Th12: second trial 12 h later. (B) Mean value of the difference in cerata extension time between the first (Tm0) and the fifth trial (Tm120) ($\Delta T = Tm0 - Tm120$) of the habituation test.

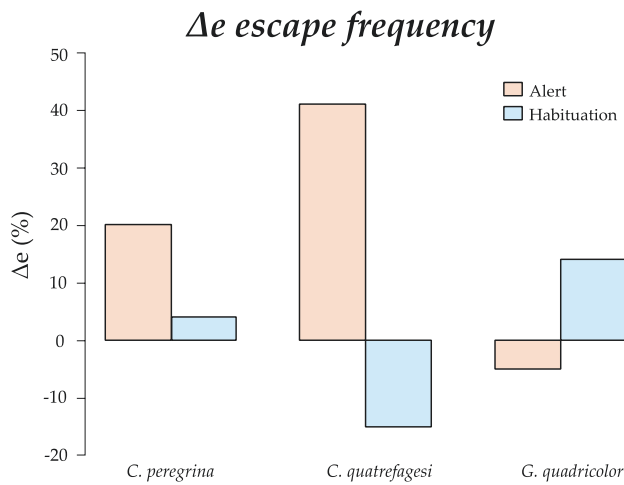


Figure 4. The difference in escape frequencies (escaping animals/ $N \times 100$) between the first and the last trial in the Alert test (Th12–Th0) and in the habituation test (Tm120–Tm0).

well represented by the component and highly correlated with each other (Table 2; SM, Figure 1). We defined the first component as the behavioral parameter for describing the “exploratory activity” of individuals. On the contrary, the second component showed a different pattern: thigmotaxis and time of habituation responses provided the larger loading to explain the second component, whereas other traits provided opposite loadings (e.g., alert response) or they did not load, that is, value closed to 0 to this component (Table 2; Figure 5). We defined the second component as the “learning ability” due to the major contribution of the habituation and alertness responses to a repeated stimulus and the spatial exploration of the environment by following the edge of arena.

By considering the rotated value from the “exploratory activity” parameter, the analysis revealed a significant effect of individual “Size” ($F_{1,41} = 6.608$, $P < 0.05$) and an effect of the “Species” ($F_{2,41} = 37.571$, $P < 0.001$). Post hoc analysis for a more detailed investigation of pairwise differences among species revealed significant differences among the native species (*C. quatrefagesi* vs. *C. peregrina*: $P < 0.001$), indicating that *C. quatrefagesi* showed reduced exploratory activity, in terms of covered distances, slower velocity and acceleration, compared with the *C. peregrina*. No differences were found when comparing those species with *G. quadricolor* ($P > 0.05$).

However, the analysis of the “learning ability” parameters highlighted an effect of “Species” ($F_{2,41} = 3.250$, $P < 0.05$). Post hoc analysis revealed that *G. quadricolor* significantly differed from both native species (*C. quatrefagesi*: $P < 0.01$; *C. peregrina*: $P < 0.01$). Given that the “learning ability” was largely explained by the thigmotaxis responses and time response of habituation (i.e., higher negative values), the invasive *G. quadricolor* showed higher values for those behavioral traits but a reduced time response of alertness, indicating that it displayed bolder phenotype compared with the native species, whereas those did not differ in their “learning ability” ($P > 0.05$). A main effect of “Size” on such parameters emerged ($F_{1,41} = 7.934$, $P < 0.01$).

Discussion

Our comparative study revealed clear behavioral and cognitive differences between sympatric invasive and Mediterranean

native nudibranch species, highlighting a possible role of exploratory activity, habituation, and alertness responses in the early stages of invasion. Specifically, the invasive *G. quadricolor* exhibited greater exploratory activity, a higher thigmotactic tendency, and an increased level of alertness, which may have potentially fostered its spread.

Although a general positive correlation between the size and all measured traits suggests individual size as an important state variable linked with the general propensity of individuals to explore, differences in locomotory activity levels between the invasive and native species were supported, with *G. quadricolor* traveling almost twice as far compared with natives, even accounting for its larger size (Figure 2). Importantly, the most significant differences in exploratory activity between the native and invasive species emerged during the introduction phase. *G. quadricolor* covered greater distances compared with both *C. peregrina* and *C. quatrefagesi*. Moreover, *G. quadricolor* showed a higher speed and acceleration compared with the native *C. quatrefagesi*. These results agree with previous studies showing that individuals on the expanding edge of the invasion front display enduring dispersal tendency and locomotor activity levels (e.g., as in the Cane toad, *Rhinella marina* Alford et al. 2009; Llewelyn et al. 2010).

G. quadricolor also showed a strong propensity for thigmotactic behavior compared with the two native species, with a notable species effect in thigmotaxis expression and size-related effect emerging in the introduction phase, whereas there were no significant differences between species in the acclimatization phase. Variation in the expression of thigmotaxis could stem from different movement and learning abilities that influence the type and amount of information collected by animals (Doria et al. 2019). Thigmotaxis is commonly considered a measure of stress during captivity, hampering cognitive performance (Harris et al. 2009). Anxiety-related thigmotaxis usually decreases with increased familiarity with the experimental housing (Simon et al. 1994; Miller et al. 2018). In contrast, we found weak differences in the proportion of time spent wall-following over the trials in *G. quadricolor*. Although thigmotaxis has not been validated as a boldness assay, it is reasonable to assume a positive relationship between open space use and boldness (Carlson and Langkilde 2013), with thigmotactic individuals enhancing the probability of success in seeking shelters (e.g., Klosinski et al. 2022), reducing at the same time the risk of being detected by natural enemies (Harris et al. 2009). It cannot be excluded that, in the early phase of an invasion process, thigmotaxis may support individual’s ability to cautiously interact with a novel environment, indirectly enhancing learning opportunities and the gathering of novel spatial information, both of which are aspects of cognitive abilities (Szabo et al. 2020). The increased level of exploration activity along with a strong propensity for thigmotactic behavior, as observed in *G. quadricolor*, may represent an effective tradeoff to reduce predation risk in the early phase of its spread, representing a possible indicator of its invasive potential.

Correlations between exploration and other proactive behaviors have been underscored in different species (e.g., Evans et al. 2010; Scales et al. 2011; Canestrelli et al. 2016), in which bolder individuals, or species, also tend to be more exploratory (Réale et al. 2010; Chapple et al. 2011; Monceau et al. 2015; Damas-Moreira et al. 2019; Burstal et al. 2020; Nordberg et al. 2021). In stable environments, proactive

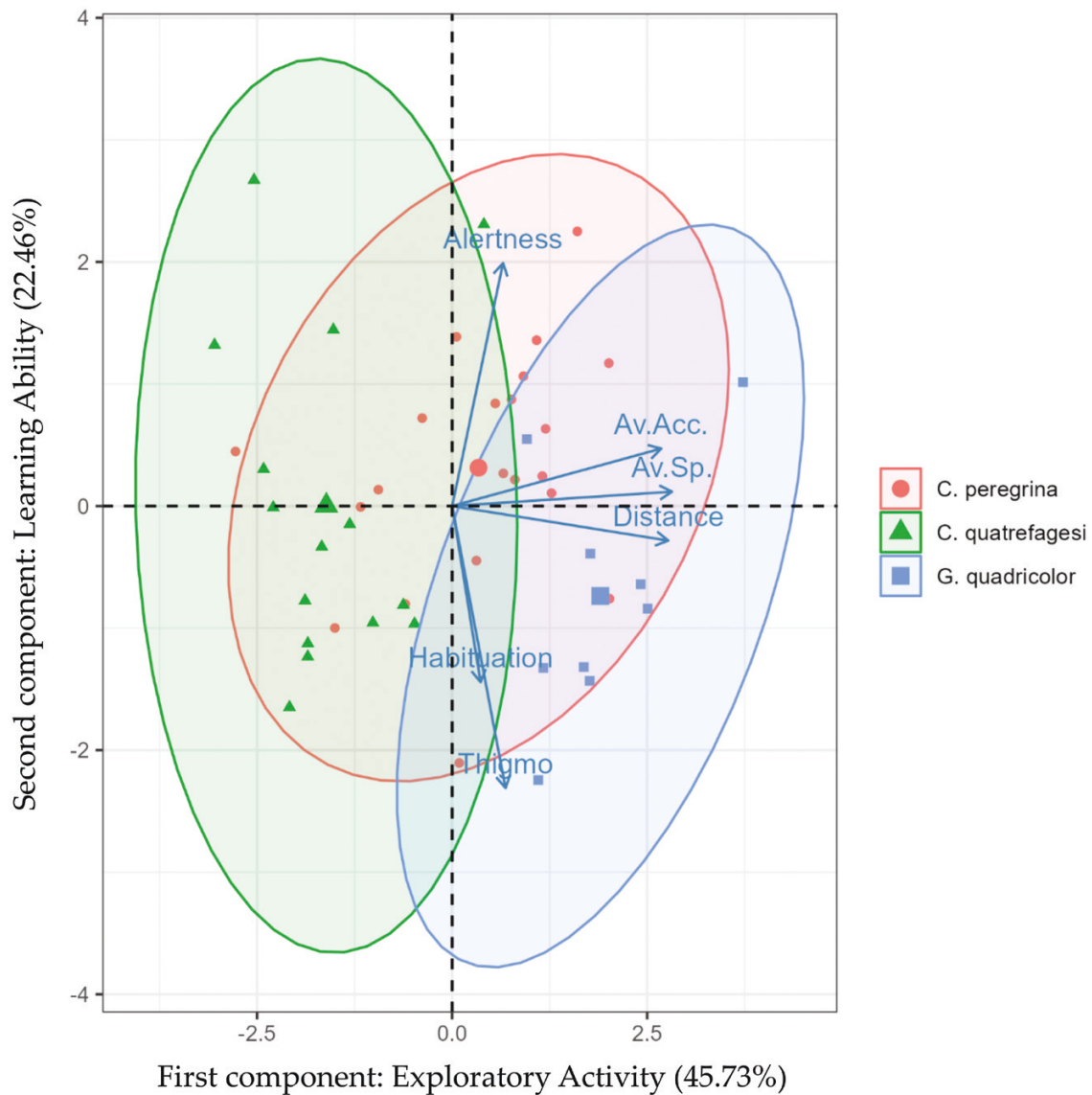


Figure 5. Biplot of the first 2 components resulted from the factorial analysis (PCA). Data represented the new rotated values for each subject clustered for the species. *Av. Sp.* = average speed; *Av. Acc.* = average acceleration; *Thigmo.* = thigmotaxis.

personalities are predicted to outcompete reactive personalities, the latter better adapting to changing environments (Koolhaas et al. 1999; Sih et al. 2004a, b). Under some circumstances, different trade-offs can be correlated to each behavioral type: proactive individuals are frequently exposed to increased predation risk (Myers and Hyman 2016; Nordberg et al. 2021). Indeed, we found that several behavioral traits associated with individual size and exploration, that is, *Distance*, *Av. Sp.*, and *Av. Acc.*, were highly correlated, suggesting the presence of high inter-individual variance of exploratory patterns in the population (Figure 5). In addition, the factor analysis highlighted how these traits were strongly and positively correlated with each other and they might be summarized as a synthetic personality trait, reflecting the exploratory propensity of individuals. The fact that the *G. quadricolor* showed the highest values might reflect its invasive ability.

G. quadricolor is a widespread invasive species, frequently recorded in Mediterranean transitional waters and lagoons (Macali et al. 2013; Furfaro et al. 2018), with locally

abundant populations, reaching densities up to 50 ind./100 m² (Furfaro et al. 2018) and it is considered a generalist species. Conversely, *C. peregrina* and *C. quatrefagesi* occupy a more specialized niche, being selective predators of hydrozoan species. Despite the consistent size difference between natives and the invader, all 3 species display garish colors and a strong propensity to stand in the open. Colorful and vulnerable prey, such as most of the nudibranch species, are often protected against predation by using chemical defenses (Aguado and Marin 2007) and, as for all aposematic species, advertised with conspicuous warning displays (Ruxton et al. 2004; Aguado and Marin 2007). Although aposematism has been shown to reduce predation in several ways (Ruxton et al. 2004), aposematic prey do not always face lower predation than non-aposematic prey (Carroll and Sherratt 2013; Seymoure et al. 2018; Yamazaki et al. 2020), being more subjected to repetitive attacks when compared with its non-aposematic mutants (Sillén-Tullberg 1985; Yamazaki et al. 2020). Conspecific aggressiveness in hunter opisthobranchs is also known, with several species, including *G. quadricolor* but

not *C. peregrina* and *C. quatrefagesi*, displaying cannibalistic behavior (Macali et al. 2023). The increased level of alertness and thigmotactic behavior observed in *G. quadricolor* (Figure 2) may arise from different selective forces acting on the expression of antipredator strategies, especially in novel environments and in an early phase of its exploration where they may be unfamiliar with local threats, while at the same time, being exposed to high levels of cannibalism. We found a similar pattern in the habituation test as well: the native species reduced their response over time, whereas *G. quadricolor* either did not change or increased its response. However, this response did not correlate with other traits (Table 2).

Being more active, with greater body size and different aposematic patterns, if compared with sympatric Mediterranean aeolids nudibranchs, *G. quadricolor* could have experienced increased predation pressure which may have promoted the sensitization of the alertness response (Figure 3B). In this framework, the continued cerata extension along with the increase of escape behavior as observed in the habituation test in *G. quadricolor* (Figure 4) may thus represent adaptive responses to novel environments continuously encountered during the invasion process. It is also interesting to note that cerata responses were moderately explained by the individual component (ICC calculated from mixed models) suggesting that personality may play an important role in the exploration of a new environment as in many vertebrate species (e.g., Bisconti et al. 2023).

Exploratory traits in both *C. peregrina* and *C. quatrefagesi* were similar and strongly correlated among each other (SM, Figure 1). Additionally, in *C. peregrina*, these traits weakly correlated with alertness, hinting at a potential phenotypic behavioral correlation in this native species. It is worth acknowledging that the observed correlation may have been influenced by the simultaneous collection of all these measures within the same test.

Behavioral traits correlations might be favored in species sharing similar ecological niches, which are negatively impacted by interspecific competition, as found in sticklebacks (Webster et al. 2009) and ants (Lichtenstein et al. 2016). However, the same was not true for *G. quadricolor*, for which we did not find any relevant correlation between traits. A lack of correlation between traits may be advantageous during the early phase of a biological invasion. If variation in behavioral traits within a population increases the likelihood of the success of an invasion front (Sih et al. 2012; Dingemans and Wolf 2013), likewise, it constrains a population, because if selection acts on one trait, correlated behaviors are also likely to be affected (Sih et al. 2012). However, we acknowledge that our sample size was relatively small to make strong inferences on behavioral (lack of) correlations.

In conclusion, our study sheds light on the role of behavior in adapting to changing environmental conditions and how behavior might contribute to success in establishing new populations in an early stage of the invasion. By comparing the behavioral profiles of both native and invasive species, we were able to gain insights on their possible impact on the ongoing success of the establishment of the South African nudibranch *G. quadricolor* in Mediterranean coastal environments. Whether these traits are the results of selective forces acting during the dispersal process or pre-existing attributes of the species remains an open question that could be addressed by testing the behavioral phenotype and plasticity in native and invasive populations of *G. quadricolor*.

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Conflict of Interest

The authors declare no conflict of interest.

Authors' Contributions

A.M. and C.C. designed research; A.M., S.F., and S.S. performed research; A.M., E.G., and S.F. analyzed data with inputs from C.C.; A.M. wrote the paper with inputs from C.C., E.G., and S.F.

Supplementary Material

Supplementary material can be found at <https://academic.oup.com/cz>.

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