Native predators living in invaded areas: responses of terrestrial amphibian species to an Argentine ant invasion

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Abstract

Predator-prey interactions play a key role in the success and impacts of invasive species. However, the effects of invasive preys on native predators have been poorly studied. Here, we first reviewed hypotheses describing potential relationships between native predators and invasive preys. Second, we examined how an invasive prey, the Argentine ant (*Linepithema humile*), affected a native terrestrial amphibian community. In the field, we looked at the structure of the amphibian community in invaded versus uninvaded areas and characterized amphibian trophic ecology. The amphibian community sampled seemed to show a species-dependent response in abundance to invasion: adults of the natterjack toad (*Bufo calamita*), the species demonstrating the highest degree of ant specialization, were less abundant in invaded areas. Although available ant biomass was significantly greater in invaded than in uninvaded areas (only Argentine ants occurred in the former), amphibians consumed relatively fewer ants in invaded areas. In the lab, we quantified amphibian consumption of Argentine ants versus native ants and assessed whether consumption patterns could have been influenced by prior exposure to the invader. The lab experiments corroborated the field results: amphibians preferred native ants over Argentine ants, and prior exposure did not influence consumption. Differences in preference explained why amphibians consumed fewer Argentine ants in spite of their greater relative availability; they might also explain why the most ant-specialized amphibians seemed to avoid invaded areas. Our results suggest the importance to account for predator feeding capacities and dietary ranges to understand the effects of invasive species at higher trophic levels.

Keywords: biotic resistance, enemy release, exotic prey naïveté, invasive prey, *Linepithema humile*.

Introduction

The vulnerability of native communities to invasions of non-native species depends on their ecological resistance, which is mainly defined by the presence of native competitors and predators (Ricciardi *et al.* 2013). Predators can promote resistance through a variety of mechanisms, including their abundance, their recognition and consumption of invasive prey, their functional response to invasive prey, and their ability to respond over time (Catford *et al.* 2009; Carlsson *et al.* 2009; Twardochleb *et al.* 2012; Carthey and Banks 2014).

For example, the well-known enemy release hypothesis (as well as its variants, such as the enemy reduction hypothesis and the enemy inversion hypothesis; Catford *et al.* 2009) states that exotic species can become invasive because they lack coevolved enemies in their introduced ranges (Keane and Crawley 2002; Colautti *et al.* 2004; Sih *et al.* 2010). In such situations, native predators do not limit the invasion, because predation does not occur or only occurs at low levels. In contrast, the more recently published exotic prey naïveté hypothesis and the increased susceptibility hypothesis posit that the naïveté of introduced prey species means they experience higher predation pressures than do native prey species (Colautti *et al.* 2004; Catford *et al.* 2009; Li *et al.* 2011; Wanger *et al.* 2011). In this case, native predators should be able to control the invasion by preying upon the introduced prey species, acting as a form of biotic resistance. The specialist-generalist hypothesis states that invasion success should be minimized when predators are generalists because they would, thus, be able to consume introduced prey (Sax *et al.* 2007; Catford *et al.* 2009). Although dietary specialization actually exists along a gradient, Catford *et al.* (2009) distinguish two extremes: the absolute generalist, which interacts with any and all species, and the absolute specialist, which preys upon a single species. However, if the invasive prey species can defend itself chemically (e.g., with toxins), then it could escape even predation by generalists. Such a situation is described by the novel weapons hypothesis—the competitive ability of invasive

prey would, therefore, be enhanced (Callaway and Ridenour 2004; Carlsson *et al.* 2009; Ricciardi *et al.* 2013).

These hypotheses are rather one-sided in that they largely focus on the success of the introduced prey species without addressing effects on native predators (but see Pintor and Byers 2015). By considering the effects on predators in each of these scenarios, we can establish a theoretical framework for understanding how invasive prey affects native predators. Here, we have expanded the hypotheses described above to address the effects of introduced prey on predators with different degrees of dietary specialization. According to the exotic prey naïveté and the increased susceptibility hypotheses, predators may benefit from the large availability of naïve prey because they can consume them (Glenn and Holway 2008; Wanger *et al.* 2011; Cabrera-Guzmán *et al.* 2012; Monzo *et al.* 2013). Based on the specialist-generalist hypothesis, generalist may benefit more than specialist predators (Maerz *et al.* 2005; Sax *et al.* 2007; Catford *et al.* 2009). And based on the enemy release hypothesis native predators would benefit more if the introduced prey species were relatively similar to native prey species (Carlsson *et al.* 2009; Robbins *et al.* 2013). Finally, according to the novel weapons hypothesis, predators will not benefit if the invasive prey releases toxins. Although native predators could develop ways for dealing with toxins, this process is expected to occur over the long term (Carlsson *et al.* 2009). Different degrees of naïveté have been observed in prey (Banks and Dickman 2007) and may also exist in native predators. In this sense, Bytheway *et al.* (2016) have shown how behavioral flexibility on the part of invasive predators can enable invaders to respond rapidly to novel situations. However, such behavioral flexibility should be less common in native predators (Carlsson *et al.* 2009): the predator may not recognize a new prey species; it may recognize an invasive species as prey but fail to capture it; or it may capture it without consuming it. Taken together, these hypotheses suggest that native predators are more likely to be negatively impacted under the

76 following conditions: they have a more specialized diet; the invasive prey species differs from
77 the native prey species included in their diets; or the invasive prey species releases toxins.

78 Ants play crucial ecological roles within ecosystems (Lach *et al.* 2010) and, consequently,
79 the negative effects of invasive ants can scale up to higher trophic levels (Holway *et al.* 2002).
80 The Argentine ant (*Linepithema humile*) is one of the five ant species included on the list of
81 100 of the world's worst invaders (Lowe *et al.* 2000; Luque *et al.* 2013). It has a remarkable
82 ability to establish itself in natural ecosystems outside of its native range; it has had striking
83 success in Mediterranean ecosystems all over the world (Suarez *et al.* 2001; Wetterer *et al.*
84 2009; Vogel *et al.* 2010). It has been used to examine a variety of ecological issues across
85 different continents (Pysek *et al.* 2008). For example, its negative effects on ant communities
86 have been studied in the greatest detail in California and Europe (e.g., Carpintero *et al.* 2005;
87 Gordon and Heller 2014). Once the Argentine ant has established itself, it displaces almost all
88 native ant species (Suarez *et al.* 1998; Carpintero *et al.* 2005; Holway and Suarez 2006;
89 Angulo *et al.* 2011). Its effects on non-ant species, including ant specialist predators, have
90 been studied in California and Japan (e.g., Suarez and Case 2002; Touyama *et al.* 2008).
91 Unlike other invasive ants (e.g., *Solenopsis invicta*, *Wasmania auropunctata*), which have a
92 venomous sting, the Argentine ant does not possess a functional stinger that it could use to
93 defend itself from predators or to subdue vertebrate prey (Holway *et al.* 2002). Although the
94 Argentine ant can prey on nestlings of some bird species, it has not been considered to be a
95 serious threat (Sockman 1997; Hooper-Bui *et al.* 2004; Suarez *et al.* 2005; Estany-Tigerström
96 *et al.* 2010, 2013). When it comes to native predators in general, negative effects related to
97 prey displacement have been observed for the ant-eating specialist *Phrynosoma coronatum*,
98 the coastal horned lizard (Suarez *et al.* 2000; Suarez and Case 2002); conversely some ant-
99 eating invertebrates appear to benefit from the presence of this invasive ant (Touyama *et al.*

2008; Glenn and Holway 2008). These contrasting results suggest that dietary specialization is not the only factor driving the impacts of the Argentine ant on predators.

In this study, we examined the threat posed by the Argentine ant to a native amphibian community. Amphibians are the world's most vulnerable group of vertebrates (accounting for ~ 41% of endangered species; Hoffmann *et al.* 2010). Terrestrial amphibians are known to consume large quantities of ants (see Online Resource 1) and are, thus, potentially vulnerable to Argentine ant invasions. We aimed to answer three key questions. First, is amphibian abundance different in invaded areas because it is dependent on dietary specialization? We predicted that the greater a species' dietary specialization on ants, the more its abundance would decrease in invaded areas. Second, is ant availability similar in invaded and uninvaded areas, and do predators with different dietary specializations track ant availability differently? We predicted that ant consumption would track ant availability for generalist predators but would decrease for the most specialized predators. Because no other ant species are available in invaded areas, predators could compensate by consuming prey of other taxa (i.e., by excluding ants from their diets). Then, if a dietary shift was to occur, the predators' nitrogen isotopic values would be expected to differ because the nitrogen isotopic value of a predator reflects that of its prey (Post 2002). Third, do amphibian ant specialists prefer native ants to Argentine ants? Is this preference affected by ant morphology or by prior exposure to the Argentine ant? We predicted that the consumption of Argentine ants by amphibian ant specialists would depend on the degree of similarity between the Argentine ant and the native ant species consumed by the specialist. We also predicted that prior exposure to the invader could alter consumption patterns in one of two ways: (a) consumption could increase relative to a naïve individual if the encounter resulted in the amphibian learning to recognize the Argentine ant as prey or (b) consumption could decrease relative to a naïve individual if the

prior exposure resulted in a negative experience and the amphibian learned to avoid eating Argentine ants.

Methods

Field study

Study Area

The field study was conducted in the Doñana Biological Reserve (37° 1' N, 6° 33' W; Doñana National Park, Spain) in an open Mediterranean scrubland containing scattered pine (*Pinus pinea*) forests and isolated cork oak trees (*Quercus suber*). One week of sampling was conducted during the summer and fall of 2009 and the winter and spring of 2010.

The reserve is home to more than 30 native ant species. The Argentine ant arrived at Doñana in the 1970s at the reserve's field station (Angulo *et al.* 2011). Given that queens are wingless and workers travel only short distances (Heller *et al.* 2008), the invasion of natural areas relies on inadvertent and sporadic transport by humans, predators, or scavengers (Carpintero *et al.* 2005). Because the species avoids the scrubland (due to its low tolerance of high temperatures and dry habitats), it is now found in individual cork oaks and pine forests (Angulo *et al.* 2011). The close association between the ants and the cork oaks is fostered by food availability. Under the cork oak canopies, a dense network of interconnected nests can be found, and ants also forage in the tree trunk and branches (Carpintero *et al.* 2005). The cork oak is a keystone species because it shelters many species against the region's hot, dry summers—the tree's canopy provides shade and the root system keeps shallower soil levels humid (Kurz-Benson *et al.* 2006)—and its location near temporary ponds results in a clear environmental gradient under the tree canopy (wetter conditions closer to the pond side and drier conditions on the opposite side of the tree).

As amphibians live around ponds, they are likely to interact with Argentine ants from invaded cork oaks. The most abundant terrestrial species are the natterjack toad (*Bufo calamita*), the western spadefoot toad (*Pelobates cultripes*), the Mediterranean treefrog (*Hyla meridionalis*), and the Iberian painted frog (*Discoglossus galganoi*) (Díaz-Paniagua *et al.* 2010). Although none of them are absolute ant specialists (Online Resource 1), we can order them according to the percentage of their diet that is represented by ants: *B. calamita* (up to 72%) > *H. meridionalis* (up to 58%) > *D. galganoi* (up to 17%) > *P. cultripes* (up to 4%).

Sampling took place in and under ten centenarian cork oaks (hereafter, tree areas), five of which had been invaded and five of which remained uninvaded by the Argentine ant. Each tree area was treated as an independent replicate. To be more certain that the amphibians studied were not experiencing both invaded and uninvaded areas, no invaded tree area was closer than 250 m to any uninvaded tree area (and vice versa). Within groups (invaded or uninvaded), tree areas were separated by at least 40 m. This distance guaranteed independence in ground and tree arthropod sampling (Angulo *et al.* 2007; Gove *et al.* 2009). We were only able to sample a limited number of trees because the National Park restricted amphibian trapping and the access to some invaded trees (because of waterbird conservation concerns) and because the Argentine ant invasion pattern is patchy.

Sampling the Abundance of Amphibian Predators

Amphibians were captured using three pitfall trap lines composed of three bucket traps each (30 x 40 cm) and a barrier of 3 m x 50 cm to guide individuals into the buckets (Fig. 1). Traps were deployed for seven days during each season and checked every three hours. Most were euthanized to examine their gut contents and to collect tissue samples for the stable isotope analyses. Samples were kept in 70% alcohol until further analyses could take place in the laboratory.

Sampling the Availability of Invertebrate Prey

Invertebrates were sampled using seven pitfall traps (200-ml PVC cups 2/3 full of soapy water) and two white traps (for flying arthropods; 25 x 50 x 10 cm trays partially filled with soapy water) placed on the ground under the tree canopy (Fig. 1). Another seven pitfall traps were attached to the tree's branches and trunk. Traps were deployed for three days per sampling period and the invertebrates, collected every day, were kept in 70% alcohol.

All invertebrates were identified to the lowest taxonomic level possible. The total number of individuals was then calculated for each taxonomic group caught for each sampling day, tree area, and sampling season. This number was used to estimate biomass: the number of individuals was multiplied by the mean mass for each taxonomic group, which was obtained by measuring the dry mass of 10–30 individuals.

Stomach Content Analyses

Predator stomach fullness (i.e., whether the stomach contained food or was empty) was determined, and the stomach contents were removed and preserved in alcohol (70%) until the prey species could be identified to the lowest taxonomic level possible using the invertebrates obtained in the pitfall traps as references. The relative importance of each prey item in a predator's diet was assessed in two ways: (1) using prey biomass: the percentage of total biomass attributable to each prey item ($100 \times [\text{biomass of a specific prey item} / \text{total biomass of all prey items}]$) and (2) prey frequency: the percentage of each prey item across all non-empty stomachs ($100 \times [\text{number of stomachs containing a specific prey item} / \text{total number of stomachs containing prey}]$). To limit bias due to digestion in the biomass calculations, the mass of the whole body of one individual was used, which was determined using the individuals obtained via pitfall trapping.

A cumulative prey curve was constructed to assess whether an adequate number of stomachs had been sampled. The order of the stomachs was randomized ten times, and the

mean ($\pm$ SE) of singleton prey items was plotted to minimize the possible bias resulting from sampling order. The point at which the prey curve approached an asymptote revealed the number of stomachs needed to accurately characterize the diet (Online Resource 2).

Isotopic Analyses

Stable isotope methods are currently among the most powerful tools used in the study of trophic relationships and animal diets. However, it is difficult to obtain exact estimates of isotopic values, as they can be affected by a number of factors (Post 2002; Vanderklift and Ponsard 2003; Caut *et al.* 2009). To reduce variability when comparing the isotopic values of amphibians captured in different areas and on different dates, it is necessary to obtain an isotopic baseline (Lorrain *et al.* 2014). Isotopic baselines are known to vary across time and space and can influence the range of nitrogen isotopic values within a given food chain at a given time. To estimate the nitrogen isotopic baseline, samples of the most abundant plant species found in the shade cast by the canopy of each tree were collected during each season and identified. The mean of their isotopic values was used as the baseline. To estimate the nitrogen isotopic values for the amphibians, liver samples were collected. Both sample types (plants and amphibian livers) were dried at 60 °C for 48 h, ground to a fine powder, weighed in tin capsules, and stored in a desiccator until isotopic analyses took place. The analyses were performed using a continuous flow isotope ratio mass spectrometry system that consisted of a Flash HT Plus elemental analyser coupled to a Delta-V Advantage isotope ratio mass spectrometer via a CONFLO IV interface (Thermo Fisher Scientific, Bremen, Germany). The system was located in the Stable Isotope Laboratory at the Doñana Biological Station (LIE-EBD; <http://www.ebd.csic.es/lie/Home.html>). Isotopic ratios are presented as δ values (‰); they are relative to atmospheric nitrogen and expressed as $\delta^{15}\text{N} = [(\text{R}_{\text{sample}}/\text{R}_{\text{standard}}) - 1] \times 1000$, where R is $^{15}\text{N}/^{14}\text{N}$. The reference material was IAEA-N1 (+0.4‰). Replicates of internal laboratory standards (which had been previously calibrated

with international standards such as IAEA-N1) were regularly included in the sampling sequence and indicated that analytical measurement errors never exceeded $\pm 0.2\%$.

Laboratory preference experiment

Fourteen adult natterjack toads (*B. calamita*) were captured in the field in spring 2013. Eight came from uninvaded areas, and six came from invaded areas. All the adults collected in uninvaded areas were captured 1.5 km away from invaded areas. It is highly unlikely that they had previous contact with the Argentine ant. In the laboratory, they were individually housed and fed mealworms, pillbugs, and small crickets *ad libitum*. The day before each preference trial, the toads were not fed.

In addition, several hundred workers were collected from colonies of the Argentine ant and from colonies of three native ant species: *Tapinoma nigerrimum*, which is similar in size and taxonomically related to the Argentine ant; *Aphaenogaster senilis*, 3-10 times larger than the Argentine ant and one of the most abundant scrubland species in our study area; and *Crematogaster scutellaris*, the most abundant species in Doñana's cork oaks (Carpintero *et al.* 2005).

Two kinds of preference tests were performed. In the first test, five workers of each ant species (for a total of 20 ants) were simultaneously added to a terrarium. A toad was then placed in the center of the terrarium, and the time at which it ate each of the ants over a 30-min period was recorded. Each toad was tested five times (N = 70 trials; 14 individuals; 1400 ants tested); trials took place at least three hours apart. In the second test, which also lasted 30 min, 20 ants of the same species were placed in a terrarium, and the time at which the toad ate the ants was recorded. Each toad was tested four times, with each of the four ant species (N = 56 trials; 14 individuals; 1200 ants tested). The order in which they experienced the species was random, and trials were separated by at least 18 hours. Both types of tests were

performed because, in the field, *L. humile* rarely co-occurs with native ants. As a consequence, amphibians will rarely have to choose between native and invasive species. However, it is nonetheless informative to quantify preferences and consumption rates under both sets of conditions.

Statistical analyses

General linear models were used to compare:

(a) The number of individuals (dependent variable) of different amphibian species found in invaded versus uninvaded areas across different seasons (independent variables). When juveniles were also found, adults and juveniles were placed in two distinct categories in the “species” variable. This differentiation between adults and juveniles reflects an important spatial constraint related to amphibian biology. Juveniles are unable to choose the ponds from which they emerge, while adults can choose where they forage and breed. The model examining overall amphibian abundance included tree area (five levels), invasion status (invaded vs. uninvaded), season (four levels), and amphibian species [six levels: *B. calamita* (adults and juveniles), *P. cultripipes*, *H. meridionalis*, and *D. galganoi* (adults and juveniles)]. We carried out separate analyses for each amphibian species (by specifying the “by” option in Proc Genmod, SAS software v. 9.2, SAS Institute 2008).

(b) Total available biomass and the percentage of available ant biomass (dependent variables) in invaded versus uninvaded areas across seasons (independent variables); the invasion-by-season interaction was also included. The models included tree area (five levels), invasion status (invaded vs. uninvaded), season (four levels), and sampling day (three levels). Thus, the totals used were for each day of each season and for each of the invaded and uninvaded tree areas.

(c) Total biomass in stomach contents and the percentage of ant biomass in stomach contents (dependent variables) for different amphibian species in invaded versus uninvaded

areas across seasons (independent variables) (note: *D. galganoi* was excluded from these analyses because of its small sample size). In this case, the sample size was the number of individual amphibians for which stomach contents could be analyzed and were not empty ($N = 95$, see Online Resource 3a). The model included amphibian species (four levels), tree area (five levels), invasion status (invaded vs. uninvaded), and season (four levels). Thus, the totals used were for each amphibian species, for each season, and for each of the invaded and uninvaded tree areas.

(d) The nitrogen isotopic baseline ($\delta^{15}\text{N}_{\text{TREE}} = \text{mean } \delta^{15}\text{N}$ of plants; dependent variable) for the different tree areas across seasons (independent variables). The model examining the baseline isotopic values included tree area (five levels) and season (four levels).

(e) The nitrogen isotopic values of amphibians ($\delta^{15}\text{N}$ of liver tissue; dependent variable) of different species in invaded versus uninvaded areas across seasons (independent variables). To standardize the comparisons, amphibian tissue $\delta^{15}\text{N}$ values were corrected using the isotopic baseline of each tree during each sampling period ($\delta^{15}\text{N}_{\text{COR}} = \delta^{15}\text{N}_{\text{LIVER}} - \delta^{15}\text{N}_{\text{TREE}}$). In this case, the sample size was the number of individual amphibians for which we obtained isotopic values ($N = 106$, Online Resource 3a). The model included amphibian species (six levels), invasion status (invaded vs. uninvaded), the invasion-status-by-season interaction, and season (four levels).

As necessary, tree area identity was included as a repeated measures factor (“repeated subject” command in Proc Genmod, SAS software v. 9.2). Models of the total stomach content biomass also included the sex of the animal as a fixed effect. The normality of all the dependent variables was tested before models were fitted. A Poisson distribution and a log-link function were used for the models of available biomass and stomach content biomass (except in the case of the total biomass of stomach contents, for which a gamma distribution with a log-link function was used); model deviance was, thus, minimized. Because one of the

invaded trees was flooded during the winter and the spring, the number of invaded trees was reduced to four.

The data from the preference experiments were analyzed using survival analysis, which estimated the probability of ants being eaten as a function of time. Mixed effects Cox models were used so that random factors could be included; we employed the `coxme` package (Therneau 2015) in the R software (R Core Team 2016). For the first test, the model included (a) ant species, to evaluate toad preference for different prey species; (b) the invasion status of the areas where the amphibians were captured to test for differences in amphibian naïveté to the Argentine ant; (c) the trial order for a given individual, to test whether learning occurred following exposure to the ants; and (d) the ant-species-by-trial-order interaction to test for differences in amphibian learning among ant species. Because learning was ant-species dependent, trial order was also tested for each ant species separately. Individual amphibian identity and trial number were included as random factors. For the second test, the model included only ant species and individual amphibian identity (as a random factor). The significance of each variable was tested using a Chi-squared test that compared the likelihood of the full model with that of the full model minus the variable of interest. In the latter model, the interaction between two variables was also removed when the significance of only one of the two variables was being tested. When significant, the model with the highest likelihood value (or the simplest model in case this value was equal) was considered the best.

Results

Amphibian Abundance in Invaded and Uninvaded Areas

Over a total of 342 trap nights, 174 amphibians of 4 different species were caught: 124 natterjack toads (*B. calamita*), 27 western spadefoot toads (*P. cultripes*), 15 stripeless tree frogs (*H. meridionalis*), and 8 Iberian painted frogs (*D. galganoi*) (Fig. 2a). All were adults,

except for most of the *B. calamita* captured in the spring (90 juveniles and 5 adults) and most of the *D. galganoi* (6 juveniles) (Online Resource 3a, Fig. 2a).

In the case of *D. galganoi* and *H. meridionalis*, the numbers of adults captured did not differ based on invasion status or season, nor was the interaction between variables significant (*D. galganoi*: $\chi^2_1 = 0.03$, $p = 0.860$; $\chi^2_3 = 2.05$, $p = 0.561$; and $\chi^2_3 = 2.05$, $p = 0.561$, respectively; *H. meridionalis*: $\chi^2_1 = 1.05$, $p = 0.306$; $\chi^2_2 = 5.05$, $p = 0.080$; and $\chi^2_3 = 3.11$, $p = 0.374$, respectively). *D. galganoi* and *B. calamita* juveniles occurred in higher numbers in invaded areas, but neither season nor the invasion-by-season interaction was significant (*D. galganoi*: $\chi^2_1 = 4.11$, $p = 0.043$; $\chi^2_2 = 4.19$, $p = 0.123$; $\chi^2_2 = 4.19$, $p = 0.123$, respectively; *B. calamita*: $\chi^2_1 = 3.92$, $p = 0.048$; $\chi^2_2 = 5.99$, $p = 0.050$; $\chi^2_2 = 3.99$, $p = 0.136$, respectively). In the case of *P. cultripes* adults, invasion status and season were marginally significant, but their interaction was not significant ($\chi^2_1 = 3.70$, $p = 0.054$; $\chi^2_3 = 7.70$, $p = 0.053$; and $\chi^2_3 = 6.83$, $p = 0.078$, respectively). *P. cultripes* was more abundant in invaded areas than in uninvaded areas and in the fall than in the winter (Fig 2a). In the case of *B. calamita* adults, invasion status was significant, while season and the invasion-by-season interaction were not significant ($\chi^2_1 = 5.67$, $p = 0.017$; $\chi^2_3 = 6.99$, $p = 0.072$; and $\chi^2_3 = 5.92$, $p = 0.116$, respectively). Fewer adults of *B. calamita* were observed in invaded areas (Fig. 2a).

Prey Availability

A total of 5319 non-ant invertebrates and 22,386 ants (mostly Argentine ants) were captured in invaded areas. In uninvaded areas, 6545 non-ant invertebrates and 4614 native ants were captured; no Argentine ants were present. Beetles and millipedes accounted for more than 40% of the available biomass across all seasons, except in the winter, when flies were more abundant than millipedes (Fig. 3a). Total available biomass was nearly significantly different across seasons but was not affected by invasion status or the invasion-by-season interaction ($\chi^2_3 = 7.48$, $p = 0.058$; $\chi^2_1 = 3.32$, $p = 0.068$; $\chi^2_3 = 6.26$, $p = 0.100$, respectively; $N = 114$).

A total of 27,000 ants were captured, of which 22,381 were Argentine ants (Online Resource 4a). In uninvaded areas, 14 ant species were found in and under trees (Fig. 3b). Only Argentine ants were found in invaded areas (except for *Temnothorax* sp., which appeared in the summer in two invaded areas), and only native ants were found in uninvaded areas. The percentage of available ant biomass differed significantly between invaded and uninvaded areas and across seasons, but the interaction between the two factors was not significant ($\chi^2_1 = 6.35, p = 0.012$; $\chi^2_3 = 8.34, p = 0.040$; and $\chi^2_3 = 6.62, p = 0.085$, respectively; $N = 114$). Ant biomass was greater in invaded versus uninvaded areas (Online Resource 4a).

Amphibian Diet in Invaded and Uninvaded Areas

Stomach contents were obtained from 112 amphibians (9.8% had empty stomachs, Online Resource 3a). However, isotopic samples were obtained from 106 individuals because the liver samples from six individuals had deteriorated. The cumulative prey curve for the two major amphibian species, *B. calamita* (adults and juveniles) and *P. cultripes*, reached a well-defined asymptote, indicating that the sample size was sufficient to adequately describe the amphibians' diets (Online Resource 2). For *H. meridionalis* and *D. galganoi*, sample sizes were lower, but the results are nonetheless provided for the sake of comparison.

Based on their stomach contents, the amphibians had varied diets; they consumed nine different taxonomic groups (Fig. 3a). In terms of total biomass, Coleoptera was by far the amphibians' most frequent prey (Fig. 3a); they made up 16-84% of their diets (except in the case of *D. galganoi*). Indeed Formicidae was the second or third most frequently consumed group (up to 37% of dietary biomass). Total consumed biomass did not differ based on invasion status, species, or season ($\chi^2_1 = 0.01, p = 0.933$; $\chi^2_3 = 2.09, p = 0.555$; and $\chi^2_3 = 5.23, p = 0.156$, respectively; $N = 95$).

Ants were found in almost all the stomachs of *B. calamita* adults and juveniles and *H. meridionalis* adults (32/34, 30/30, and 9/12, respectively; Online Resource 3a). Formicidae

was less common in *P. cultripes* stomachs (both in terms of biomass and frequency). Ant frequency, mean percentage of consumed ant biomass, and the number of ant species consumed were greater for *B. calamita* (adults and juveniles) than for other species (Fig. 3a, Online Resource 4b). Thus, of the amphibians studied, *B. calamita* showed the greatest degree of ant specialization. Thirteen species of Formicidae were observed in the stomach contents: 12 native species and the Argentine ant (Online Resource 4b). Except in one individual, Argentine ants were the only ant species found in adult amphibians from invaded areas. Conversely, except in one individual, Argentine ants were completely absent from the stomachs of amphibians from uninvaded areas (Fig. 3b). Invasion status did have a significant effect on the percentage of ant biomass consumed ($\chi^2_1 = 5.04$, $p = 0.025$, $N = 95$). Significantly more ant biomass was consumed in uninvaded areas than in invaded areas (8.15 ± 2.3 g versus 0.86 ± 0.2 g, respectively). Season and species did not have an effect ($\chi^2_3 = 1.87$, $p = 0.600$, and $\chi^2_3 = 1.98$, $p = 0.577$, respectively; $N = 95$).

The nitrogen isotopic baseline was significantly different across seasons and individual tree areas ($\chi^2_3 = 25.43$, $p < 0.001$ and $\chi^2_9 = 18.11$, $p = 0.034$, respectively; $N = 362$, Online Resource 3b). This finding meant that the amphibians' nitrogen isotopic values needed to be corrected. Amphibian nitrogen isotopic ratios did not differ between invaded and uninvaded areas, among species, or across seasons (invasion status: $\chi^2_1 = 3.10$, $p = 0.078$; amphibian species: $\chi^2_5 = 7.52$, $p = 0.185$; season: $\chi^2_3 = 4.44$, $p = 0.218$; $N = 106$); the interaction between invasion status and species was not significant ($\chi^2_4 = 3.05$, $p = 0.549$, $N = 106$) (Fig. 2b). The values were highly variable, which probably explains why no effect of invasion status was found.

Preference Tests

Similar results were obtained from the two types of preference tests (providing the adult toad with four ant species simultaneously or each ant species separately). *B. calamita* adults ate

both native ants and Argentine ants. However, they ate native ants faster and in greater quantities (Fig. 4a). When the amphibians were simultaneously offered the four ant species, there were ant-species-specific differences in consumption ($\chi^2_3 = 406.34$, $p < 0.0001$, $N = 1400$). Fewer Argentine ants were eaten: at 30 min, around 50% of Argentine ants were left versus fewer than 30% of native ants (Fig. 4a). When we compared survivorship, the Argentine ant survived longer than the native ants: 2.03 times longer than *C. scutellaris*, 5.17 times longer than *A. senilis*, and 5.42 times longer than *T. nigerrimum*. When the amphibians were offered one ant species at a time, there were again ant-species-specific differences in consumption ($\chi^2_3 = 146.72$, $p < 0.0001$, $N = 1120$): 30% of Argentine ants remained at 30 min versus less than 20% of native ants. Once again, the Argentine ant survived longer than the native ants: 2.31 times longer than *C. scutellaris*, 2.59 times longer than *T. nigerrimum*, and 2.78 times longer than *A. senilis*. Furthermore, in the second test, no ants were eaten in six of the trials; the percentage of trials in which no ants were eaten was 21.4% for the Argentine ant (3 trials), 14.3% for *T. nigerrimum* (2 trials), 7.1% for *C. scutellaris* (1 trial), and 0% for *A. senilis*.

Amphibians from invaded versus uninvaded areas did not differ in their rates of Argentine ant consumption ($\chi^2_1 = 6e-04$, $p = 0.981$, $N = 1400$ observations, Fig. 4b). Both trial order and the ant-species-by-trial-order interaction were significant, meaning that there was a learning process and a significant difference among ant species in the relative degree of learning ($\chi^2_1 = 37.81$, $p < 0.0001$; $\chi^2_3 = 12.831$, $p = 0.005$, respectively, $N = 1400$ observations). Toads fed on each ant species faster in subsequent trials (*L. humile*: $\chi^2_1 = 22.94$, $p < 0.001$, Fig. 4c; *A. senilis*: $\chi^2_1 = 34.06$, $p < 0.001$; *T. nigerrimum*: $\chi^2_1 = 20.10$, $p < 0.001$; *C. scutellaris*: $\chi^2_1 = 28.82$, $p < 0.001$; $N = 350$).

Discussion

Although none of the terrestrial amphibians in Doñana National Park exclusively consume ant, ants do constitute a significant percentage of their diets. Indeed, when we considered the relative representation of ants in amphibian diets, as compared to other invertebrate taxa, we found that amphibians in invaded areas consumed less ant biomass than amphibians in uninvaded areas, even though more ant biomass was available in invaded areas (but comprised only Argentine ants). These differences in consumption could be the result of a preference for native ants over Argentine ants (even post exposure). Because levels of available and consumed invertebrate biomass were similar between invaded and uninvaded areas and amphibians in invaded areas consumed less ant biomass, amphibians shifted to non-ant prey in invaded areas. The Argentine ant invasion also seems to have differentially affected the abundances of adult amphibians. While *H. meridionalis* and *D. galganoi* appeared to be unaffected, *P. cultripes* was more common in invaded areas, although this difference was less pronounced in the winter than in the fall. In contrast, *B. calamita*, the greatest ant specialist in the amphibian community, seemed to avoid invaded areas.

Effects on the Amphibian Community

The Argentine ant is already established in some suitable habitats in Doñana, where it has replaced most native ant species by competition (Carpintero *et al.* 2005, 2007; Angulo *et al.* 2011). According to the best known hypotheses that examine the potential relationships between invasive prey and native predators (Callaway and Ridenour 2004; Catford *et al.* 2009; Carlsson *et al.* 2009; Sax *et al.* 2007; Ricciardi *et al.* 2013), native predators are more likely to be negatively impacted if they display greater dietary specialization. Our stable isotopic analyses confirm that Doñana's terrestrial amphibian community displayed less dietary specialization than expected: $\delta^{15}\text{N}$ liver tissue values were highly variable even though the nitrogen isotopic baseline was relatively stable (Post 2002; Vanderklift and Ponsard

2003). However, ants may nonetheless make up a significant percentage of their diets (the percentage of consumed ant biomass was much greater than the percentage of available ant biomass).

To date, Argentine ant invasions had only been found to reduce predator abundance in the case of the coastal horned lizard, *P. coronatum*, a highly specialized predator of ants (Suarez *et al.* 2000; Fisher *et al.* 2002). In contrast, other ant specialists seem to have benefitted from the abundant food that stems from Argentine ant invasions (Touyama *et al.* 2008; Glenn and Holway 2008). In this study, we found differential effects of the invasion on adult amphibian abundance, which could be related to the species' degree of dietary specialization. The amphibian that consumed the smallest percentage of ants, *P. cultripes*, has the highest abundance of adults in invaded areas. However, there were no differences in adult abundance between invaded and uninvaded areas for *H. meridionalis* and *D. galganoi*, species that consumed intermediate percentages of ants. In contrast, we discovered that *B. calamita* adults were less abundant in invaded areas. This finding makes sense, given that *B. calamita* (as well as other bufonids; Isacch and Barg 2002) is the greatest ant specialist of the four amphibian species studied. Furthermore, in our study, the number of ants consumed, the mean percentage of ant biomass consumed, and the number of ant species consumed were greatest for *B. calamita*. However, *B. calamita* juveniles occurred in higher numbers in invaded areas than in uninvaded areas. Similar results were seen for *G. galganoi* juveniles. This contrast between adults and juveniles could be explained by the fact that juveniles are unable to choose the ponds from which they emerge, while adults can choose where they forage and breed. The greater abundance of *P. cultripes* adults in invaded areas was counterintuitive. It might be that they were attracted by the greater ant biomass in those areas or by a lower degree of interspecific competition, as other amphibian species seemed to avoid these areas (i.e., *B. calamita* adults). Although these results should be interpreted with caution because of

our low amphibian sample sizes and given the difficulties associated with estimating amphibian abundance (i.e., terrestrial amphibians strongly respond to fluctuations in precipitation), we propose that Argentine ant invasions may have an effect on the most ant-specialized amphibian species.

Dietary Shifts in the Presence of the Argentine Ant

Predators may not consume invasive prey if they are naïve, if they are absolute specialists (as per Catford *et al.* 2009), or if invasive prey release toxins. As a consequence, dietary shifts and reductions in prey availability occur, which are some of the mechanisms that explain the negative effects invasive prey species have on predators (Suarez *et al.* 2000; Suarez and Case 2002; Caut *et al.* 2008). In less extreme scenarios, predators should consume large quantities of the invasive prey, at least according to the exotic prey naïveté or increased susceptibility hypotheses; such may also be the case if the predator is an absolute generalist (Colautti *et al.* 2004; Catford *et al.* 2009; Li *et al.* 2011; Wanger *et al.* 2011). The amphibian community we studied here seems to provide an example of a less extreme scenario, as Argentine ants were consumed to some degree. However, the diets of Doñana amphibians clearly reflect the previously described (Angulo *et al.* 2011) negative effects of the Argentine ant on native ant communities: amphibians from uninvaded areas consumed a greater diversity of native ants, which largely corresponded to species availability. In contrast, the Argentine ant was almost the only ant species found in the stomach contents of amphibians from invaded areas, which also corresponded to species availability. Even if certain amphibians, such as *B. calamita* adults, seemed to avoid invaded areas, individuals may remain in them long enough to consume an entire meal of Argentine ants. Adult amphibians can have large foraging areas (Miaud *et al.* 2000), but we only observed two cases in which individuals ate ants that did not correspond to the area in which they were captured.

Previous work at our study site has shown that amphibians include the Argentine ant in their diets (Díaz-Paniagua *et al.* 2005). Indeed, the Argentine ant appears to be consumed by most ant predators, including amphibians (Ito *et al.* 2009), jumping spiders (Touyama *et al.* 2008), and pit-building ant lions (Glenn and Holway 2008). In nature, the coastal horned lizard does not consume the Argentine ant and compensates for the elimination of its main prey species (native ants) by consuming greater quantities of other invertebrates (Suarez *et al.* 2000; Suarez and Case 2002). In our study, we found that amphibians consumed significantly smaller percentages of ant biomass in invaded areas than in uninvaded areas. Clearly, amphibians do not completely replace native ants by Argentine ants, even when levels of Argentine ant biomass are higher than those of native ants. Doñana amphibians compensated for the lack of native ants by shifting their diet to include other invertebrates: the total biomass consumed was similar in invaded and uninvaded areas. Because the percentage of ants consumed was lower in invaded areas, the percentage of other invertebrates consumed should be higher.

Amphibian Prey Preferences

When native predators are faced with novel prey, they may fail to recognize or capture the introduced prey species or may be unable to consume it because it is unpalatable or contains toxins. We found that *B. calamita* adults recognized Argentine ants as prey, capturing and consuming them, albeit at markedly lower rates than for native ants. This result could stem from lower detection probabilities or lesser palatability resulting from the Argentine ant's small size or color, as seen in the case of the coastal horned lizard (Suarez *et al.* 2000). Of the ants tested in the laboratory, the Argentine ant was the smallest, followed by *T. nigerrimum* and *C. scutellaris*; *A. senilis* was the largest. *A. senilis* and *T. nigerrimum* are black, *C. scutellaris* is two toned (black and red), and the Argentine ant is sand colored. Thus, although the Argentine ant is most similar to *T. nigerrimum*, their survivorship patterns in the

519 preference tests differed dramatically. The Argentine ant's marked dissimilarity in size and
520 color might explain its higher survival rates in the laboratory experiment. In the field, of the
521 12 native ant species consumed by Doñana amphibians, only one (*Plagiolepis schmitzii*) is
522 smaller than the Argentine ant (Arnan *et al.* 2014). Although prey movement is required to
523 trigger feeding responses in some anurans (Oliver 1955), Doñana amphibians consumed
524 native ants that moved faster (*A. senilis*) and slower (*C. scutellaris* and *Temnothorax* sp.) than
525 the Argentine ant, which suggests that movement does not play a significant role. The
526 preference for native ants could be explained by the Argentine ant having a lower energetic
527 value. However, Pekár and Mayntz (2014) recently showed that differences in the nutritional
528 composition of European ants cannot fully explain the preferences of predators. Finally, even
529 if the Argentine ant has antipredatory defenses, such as aggressive behavior or noxious
530 chemicals (Suarez and Case 2002; Glenn and Holway 2008; Robbins *et al.* 2013), they did not
531 alter the response of *B. calamita* toads, which increased their feeding response with greater
532 exposure. It could be that learning is occurring. Robbins *et al.* (2013) also showed that fence
533 lizards learned to eat invasive ants over successive feeding trials. However, in our study,
534 adults of *B. calamita* from invaded and uninvaded areas consumed Argentine ants at similar
535 rates, indicating that prior exposure neither positively nor negatively influenced consumption.

536 In conclusion, when it comes to interactions between native predators and invasive prey, it
537 is essential to consider both the direct and indirect effects of invaders on the native predator
538 community, which means examining predator diets, prey availability, and predator feeding
539 capacities (e.g., prey preferences, ability to learn). Although many generalist predators
540 include ants in their diets, detailed studies on how predators are affected by Argentine ant
541 invasions are very limited. Research on such bottom-up effects is important if we are to
542 understand the impact of ecologically important invaders at higher trophic levels.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical approval

All applicable institutional and national guidelines for the care and use of animals were followed.

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Figure Legends:

Fig. 1 (a) A picture of a cork oak (*Quercus suber*) and (b) schematic of the trapping protocol for a given tree. The pitfall traps shown inside the tree trunk were attached to the trunk and branches

Fig. 2 (a) Number of amphibians captured (mean $\pm$ SE) and (b) the $\delta^{15}\text{N}_{\text{COR}}$ values of amphibian livers (mean $\pm$ SE) for invaded and uninvaded areas (INV in black and UNI in white, respectively) across different seasons (SU = summer, FA = fall, WI = winter, and SP = spring). Data for adults and juveniles are separated (juveniles are specified with “juv.”). Only seasons for which abundance was greater than zero in at least one tree area are represented

Fig. 3 Mean percentage of (a) invertebrate biomass and (b) ant species biomass (other = ant species < 5 % of relative ant biomass). The figures represent relative availability as estimated from pitfall traps ( ) and relative presence in amphibian stomach contents for the different seasons in invaded (INV) versus uninvaded (UNI) areas. In (b), the numbers above the bars indicate the percentage of ant items out of all the invertebrates found in amphibian stomachs

Fig. 4 Ant preferences demonstrated by *Bufo calamita*. Consumption of live ants over the course of the first trial (simultaneous exposure to four ant species): (a) by each ant species; (b) for Argentine ants exposed to *B. calamita* adults from invaded versus uninvaded areas; and (c) by trial order (1st to 5th; all ants). Shaded areas represent 95% CI

Electronic supplementary material.

The online version of this article (doi: 10.1007/s00442-017-3929-x) contains supplementary material, which is available to authorized users.

Online Resource 1—Formicidae in the diet of four Mediterranean amphibian species

Online Resource 2—Cumulative prey curve for *B. calamita* and *P. cultripes*

Online Resource 3—Nitrogen isotopic values of the amphibians captured and the plant species sampled (used as baseline)

Fig. 1

Fig. 2

Fig. 3

Fig. 4