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Contributed Papers

Effects of the Argentine ant venom on terrestrial amphibians

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Abstract: Invasive species have major impacts on biodiversity and are one of the primary causes of amphibian decline and extinction. Unlike other top ant invaders that negatively affect larger fauna via chemical defensive compounds, the Argentine ant (*Linepithema humile*) does not have a functional sting. Nonetheless, it deploys defensive compounds against competitors and adversaries. We estimated levels of ant aggression toward 3 native terrestrial amphibians by challenging juveniles in field ant trails and in lab ant foraging arenas. We measured the composition and quantities of toxin in *L. humile* by analyzing pygidial glands and whole-body contents. We examined the mechanisms of toxicity in juvenile amphibians by quantifying the toxin in amphibian tissues, searching for histological damages, and calculating toxic doses for each amphibian species. To determine the potential scope of the threat to amphibians, we used global databases to estimate the number, ranges, and conservation status of terrestrial amphibian species with ranges that overlap those of *L. humile*. Juvenile amphibians co-occurring spatially and temporally with *L. humile* die when they encounter *L. humile* on an ant trail. In the lab, when a juvenile amphibian came in contact with *L. humile* the ants reacted quickly to spray pygidial-gland venom onto the juveniles. Iridomyrmecin was the toxic compound in the spray. Following absorption, it accumulated in brain, kidney, and liver tissue. Toxic dose for amphibian was species dependent. Worldwide, an estimated 817 terrestrial amphibian species overlap in range with *L. humile*, and 6.2% of them are classified as threatened. Our findings highlight the high potential of *L. humile* venom to negatively affect amphibian juveniles and provide a basis for exploring the largely overlooked impacts this ant has in its wide invasive range.

Keywords: amphibian decline, chemical weapons, invasive species, impact prioritization, *Linepithema humile*, predator–prey relationships

Efectos del Veneno de la Hormiga Argentina sobre los Anfibios Terrestres

Resumen: Las especies invasoras tienen un impacto importante sobre la biodiversidad y son una de las causas principales del declive y extinción de los anfibios. A diferencia de otras hormigas super-invasoras que afectan negativamente a animales más grandes por medio de compuestos químicos de defensa, la hormiga argentina (*Linepithema humile*) no tiene unaguijón funcional. Sin embargo, esta hormiga despliega compuestos defensivos contra sus competidores y adversarios. Estimamos los niveles de agresión de las hormigas hacia tres anfibios terrestres nativos exponiendo a los anfibios juveniles en pistas de hormigas en el campo y en las arenas de forrajeo de las hormigas en el laboratorio. Medimos la composición y las cantidades de toxina que presenta *L.*

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humile por medio del análisis de las glándulas pigidiales y el contenido en el cuerpo completo. Examinamos los mecanismos de la toxicidad en los anfibios juveniles cuantificando la toxina en el tejido del anfibio, buscando daños histológicos y calculando las dosis tóxicas para cada especie de anfibio. Para determinar el alcance potencial de la amenaza para los anfibios usamos bases de datos mundiales para estimar el número, distribución y estado de conservación de las especies terrestres de anfibios con distribuciones que se solapan con la de *L. humile*. Los anfibios juveniles que co-ocurren temporal y espacialmente con *L. humile* mueren al encontrarse con esta especie de hormiga en sus pistas. En el laboratorio, cuando un anfibio juvenil entró en contacto con *L. humile*, las hormigas reaccionaron rápidamente rociando a estos juveniles con veneno proveniente de las glándulas pigidiales. La iridomyrmecina fue el compuesto tóxico que encontramos en las glándulas pigidiales. Después de ser absorbida por la piel del anfibio, se acumuló en el cerebro, los riñones y el hígado. La dosis tóxica para los anfibios depende de la especie. A nivel mundial, se estima que 817 especies de anfibios terrestres tienen una distribución que se solapa con la de *L. humile*, y el 6.2% de estas especies se encuentran clasificadas como amenazadas. Nuestros hallazgos resaltan el potencial alto del veneno de *L. humile* para tener efectos negativos sobre los anfibios juveniles y también proporcionan una base para la exploración de los impactos de esta hormiga en su amplio rango invasivo, los cuales generalmente son ignorados.

Palabras Clave: armas químicas, declinación de anfibios, especies invasoras, priorización de impactos, relaciones depredador-presa, *Linepithema humile*

摘要: 生物入侵对生物多样性有着重要影响, 同时也是导致两栖动物数量减少和灭绝的主要原因之一。不同于其它通过化学防御物质对大型动物产生负面影响的入侵蚁类, 阿根廷蚁 (*Linepithema humile)* 不具备功能性的刺, 但它还是可以利用防御化合物来对付竞争者和攻击者。我们通过在野外蚁道和实验室的蚂蚁觅食场所观察蚂蚁如何挑战三种本土陆生两栖动物的幼体, 估计了蚂蚁的攻击性强度。通过分析阿根廷蚁臀板腺体和全身的物质组成, 我们测定了其毒素的成分和含量。通过定量两栖动物幼体组织中的毒素, 寻找组织损伤, 并计算毒素对每种两栖动物的毒性剂量, 我们分析了蚂蚁毒素对两栖动物幼体的毒性机制。接下来, 为了确定两栖动物面临潜在威胁的范围, 我们利用全球数据库分析了与阿根廷蚁分布范围重合的陆生两栖动物的数量、范围和濒危情况。在野外, 与阿根廷蚁时空上共存的两栖动物幼体在蚁道上遇到阿根廷蚂蚁时会发生死亡。在实验室中, 当两栖动物幼体接触到阿根廷蚁时, 蚂蚁会迅速做出反应, 向其喷射臀板腺体毒液。喷射液体中的有毒化合物为阿根廷虹臭蚁素, 动物吸收后会在脑、肾和肝组织中积累, 毒素对两栖动物的毒性剂量取决于物种。在世界范围内, 估计有 817 种陆生两栖动物的分布区与阿根廷蚁分布区重叠, 其中有 6.2% 为濒危物种。本研究的发现强调了阿根廷蚁的毒液对两栖动物幼体潜在的巨大负面影响, 并为探索阿根廷蚁在其广大的入侵范围内尚未得到重视的影响提供了基础。【**翻译: 胡怡思; 审校: 聂永刚**】
阿根廷蚁毒液对陆生两栖动物的影响

关键词: 两栖动物减少, 化学武器, 入侵物种, 影响优先排序, 阿根廷蚁 (*Linepithema humile)*, 捕食者-被捕食者关系

Introduction

Amphibians are the most threatened vertebrate taxa worldwide, and 41% of species are at risk of extinction (https://www.iucnredlist.org/). Since the 1980s, amphibian population declines and extinctions have outpaced those of mammals and birds (Stuart et al. 2004). Habitat alterations and disease and their synergistic effects with climate change are key drivers of extinction (Kiesecker et al. 2001; Hof et al. 2011). Overwhelmingly, study results suggest that global amphibian losses are the result of complex interactions among multiple factors acting at local scales in a context-dependent manner (Blaustein & Kiesecker 2002; Grant et al. 2016). Much of the observed decline is still attributed to "enigmatic decline" (Stuart et al. 2004); thus, quantifying lesser known threats to amphibians is important for developing effective conservation strategies.

Invasive species are a major cause of amphibian extinctions, through competition, hybridization, disease transfer, and predation (Kats & Ferrer 2003). Invasive ants, three species of which are among the world's worst invaders, have negative consequences for wildlife, including many amphibian species, due to their opportunistic predation, poisoning, or toxicity (Holway et al. 2002). For example, the red imported fire ant (*Solenopsis invicta*) negatively affects native herpetofauna, birds, and mammals (Allen et al. 2004). Its venom is normally injected by stinging and may induce anaphylaxis and, at higher doses, paralysis and death (Attygalle & Morgan 1984).

Chemical defense has evolved in ants and other social insects to protect their nests. Ants exhibit a plethora of chemicals with a clear evolutionary pathway, and they range from proteinaceous pain-inducer venom to low molecular organic toxins (Attygalle & Morgan 1984). In addition to their primary defensive role, they can, due to their toxicity, act to subdue potential prey. They also often act alone or in combination with volatile substances as alarm pheromones to elicit aggression and recruit aggressors (Blum 1996).

This is well exemplified in 1 of the 5 most invasive ants, Argentine ant (*Linepithema humile*). Although *L. humile* lacks visible weapons (e.g., a functional stinger or large mandibles), it produces substances that include volatile alarm pheromones and defensive allomones (Cavill et al. 1976). Welzel et al. (2018) established that it deploys its defensive compounds against native ants. Although indirect effects on vertebrates are also known, such as contributing to the decline of the horned lizard (*Phrynosoma coronatum*) (Suarez & Case 2002) and the spatial shift in habitat use of amphibians (Alvarez-Blanco et al. 2017), direct effects (i.e., capacity to subdue vertebrates), which could explain some of the reported indirect effects, have not been demonstrated.

We estimated levels of ant aggression directed at different amphibian species in the field and laboratory and quantified the toxin used. To determine the potential scope of the threat faced by amphibians, we used global databases to estimate the number of terrestrial amphibian species whose ranges and habitats overlap those of the Argentine ant, highlighting particularly those species listed as threatened the International Union for Conservation of Nature (IUCN 2018). Ranges and categories of the IUCN Red List are a global standard for conservation studies, ensuring consistency across taxa and regions (Betts et al. 2020). We sought to determine how hazardous the venom is to amphibians because the ant's global distribution and extensive overlap with endangered amphibian species could have serious implications for amphibian conservation.

Methods

Local study Site and Amphibian Species

The Doñana Biological Reserve (RBD) (Spain, 36°59.491'N, 6°26.999'W) hosts both terrestrial amphibians and the invasive *L. humile* (Díaz-Paniagua et al. 2010; Alvarez-Blanco et al. 2017). We collected individuals of the 3 most abundant amphibian species: natterjack toads (*Epidalea calamita*), Mediterranean treefrogs (*Hyla meridionalis*), and western spadefoot toads (*Pelobates cultripes*) (detailed methods in Supporting Information). We collected newly emerged juvenile amphibians near ponds or tadpoles that we then raised to metamorphosis. Juveniles were housed in groups in terraria and placed individually in smaller containers during trials. To compare the effects of *L. humile* on amphibians with those inflicted by other ant species, we selected two abundant co-occurring native ant species: *Aphaenogaster senilis* (Myrmecinae) and the closely related *Tapinoma* cf. *nigerrimum* (Dolichoderinae) (Arnan et al. 2012). Both ants are generalist feeders that scavenge on animal and plant remains (Arnan et al. 2012), similar to the Argentine ant. Experimental procedures were approved by the national authorities (CEBA-EBD 11–36, CEBA-EBD 11–36b, CSD2008-00040, 1043/MDCG/mect, 014-1073-00000613-FQH/MDCG/mect). Personal authorization to carry out animal experimentation was given by the Spanish MAGRAMA (CAP-T-0220-15 and EXP-000261 to P.A.-B., and CAPT-0224-15 to E.A.).

Spatial and Temporal Ant and Juvenile Amphibian Activity

During the period when newly metamorphosed *E. calamita* emerge from ponds, we established two plots separated by 400 m that encompassed invaded and uninvaded areas surrounding ponds. For two consecutive days, we placed bait (water-diluted honey and cookie on 10 pairs of plastic spoons) to attract ants along a 35-m transect and then recorded the number and species of ants and counted the number of toadlets in a 1 × 50 m transect throughout the day (0900, 1230, 1600, 1930, and 2300).

To demonstrate whether emerging amphibians feed on ants or *L. humile* preyed on them, we inspected, preliminarily, relatively permanent *L. humile* trails near the ponds and found dead amphibians on these trails. Subsequently, for 4 days/year over 3 years, we counted the number of dead juvenile amphibian along 40 m × 40 cm trails of *L. humile*.

Trail and Foraging-Arena Exposure Experiments

We sought to determine why juvenile amphibians did not escape from *L. humile* trails and whether native ants were similarly aggressive toward juveniles. We simulated ant-amphibian encounters experimentally in the field by placing *P. cultripes* and *H. meridionalis* juveniles 3 cm away from trails of the three above-mentioned ant species. Amphibians were in perforated cages (8 × 8.5 × 3 cm, mesh 5 × 5 mm) that allowed the entrance of large *A. senilis* and plastic Petri dishes (5.5 cm × 1.4 cm, with mesh 4 × 4 mm) that allowed the entrance of *T.* cf. *nigerrimum* and *L. humile* (Supporting Information). Following initial contact with the ants, the amphibians were kept in place for 2 additional minutes and then released by carefully removing the cage. They were then observed for 10 minutes or until they had moved at least 1 m away from the trail. During these 10 minutes individuals acted normally tried to escape or defend themselves from the ants, or were paralyzed. Paralyzed individuals either recovered or died. All individuals were subsequently observed in the laboratory for 48 hours to monitor their recovery. Individuals that were unaffected, escaped, or were not paralyzed were classified as alive. Those that recovered after initial paralysis were classified as paralyzed, whereas those that died within 48 hours were classified as dead.

In laboratory assays, juveniles of *P. cultripes*, *E. calamita*, or *H. meridionalis* were introduced individually into the foraging arenas of colonies of each of the 3 ant species for a maximum of 10 minutes ($n = 5$ colonies/ant species; colony details in Supporting Information). We measured the elapsed time to discovery of the juvenile by the ants and the maximum number of ants on it. In cases of apparent harmful effects to the juveniles (individual remained immobile or paralyzed for 1 minute or was being dragged off by ants) trials were stopped before 10 had minutes elapsed. After 48 hours of observation, individuals were classified as alive, paralyzed, or dead.

Histological and Chemical Differences Between *L. humile* and *T.* cf. *nigerrimum*

To determine whether *L. humile* uses a chemical attack, we compared the histology of all abdominal exocrine glands of *L. humile* and *T.* cf. *nigerrimum*. Ant gasters were fixed in 2% glutaraldehyde (buffer: 0.05 M Na-cacodylate and 0.15 M saccharose), postfixed in 2% osmium tetroxide, and embedded in Araldite (Agar Scientific, Stansted). Semithin sections (thickness of 1 μm) were created with a Leica ultramicrotome (EM UC6, Leica, Wetzlar) and stained with methylene blue and thionin. These sections were then viewed and photographed under a microscope (BX-51, Olympus, Tokyo). We examined the sections to identify all known glands and to look for previously undescribed glands.

We compared the chemical composition of the pygidial gland of the two species. We dissected the pygidial glands of five freeze-killed ants of each species immediately after death and extracted them in hexane for 24 h. We achieved compound identification via gas chromatography coupled with mass spectrometry (GC-MS) with an HP-5MS capillary column temperature programed from 60 °C (1 minute hold) to 320 °C at a rate of change of 10 °C/minutes. For iridomyrmecin quantification, extracts of 50 whole ants (10/colony) were used rather than dissected glands to avoid possible spillage during dissection. Decyl alcohol (99%) was used as the internal standard. Samples were quantified by gas chromatography as described above. Calibration curve was established using synthetic iridomyrmecin (Chauhan & Schmidt 2014; Supporting Information).

Iridomyrmecin-Exposure Experiment

To test iridomyrmecin's toxicity, we applied the synthetic compound to the backs of *P. cultripes* toadlets (isomers 1 and 2 with a ratio of 1.5:1). We exposed 10 toadlets to each of three doses of iridomyrmecin dissolved in hexane: 0.1 mg, 1 mg, and 5 mg/toadlet and pure hexane as control. Doses were calculated from Choe et al. (2012) estimations to match naturally occurring concentrations the amphibian would encounter in the field. To avoid skin irritation by the hexane solvent, solutions were applied to cavity slides, where the solvent was allowed to evaporate, and the slides were rubbed onto the toadlets' backs. After 48 h of observation, individuals were classified as alive (not affected), paralyzed (recovered from initial paralysis), or dead.

Dose–Response Experiment

To assess the number of ants necessary to elicit an effect, we constructed dose-response curves for each ant species and each amphibian species. The number of amphibians was limited to that necessary to obtain adequate dose-response curves (Supporting Information).

Doses of the toxin were obtained from a different number of either *L. humile* or *T.* cf. *nigerrimum* workers that were macerated in a ceramic bowl with 0.2 mL of dechlorinated water. A single dose of the mash was immediately applied to the back of an amphibian. After 10 minutes, the individual was gently bathed in dechlorinated water to remove the mash, and we examined the individual for neurological damage. An individual was considered affected by the toxin if an abnormal reaction was displayed in motor response, photopupillary reflex, or palpebral reflex (Supporting Information).

Physiological Effects on Juvenile Amphibians

To elucidate the venom's mechanism of action and confirm that the damage was caused by iridomyrmecin, we euthanized the amphibians used in the dose–response experiment after clinical evaluation. Half the amphibians were used to quantify iridomyrmecin levels in tissues. Animal brains, livers, and kidneys were removed and individually extracted in hexane for gas chromatography-flame ionization detector analyses.

The other half were used in histological analyses. Individuals were fixed in formalin and their livers and kidneys were removed. Tissue samples were embedded in paraffin, sectioned at a thickness of 6 μm with a Leica RM 2155 microtome, and mounted on glass slides. Sections were dewaxed through a series of xylene and ethanol washes (from 100% solution to 100% H_2O), stained with hematoxylin and eosin, rehydrated through a series of ethanol washes (from 70% to 100% solution to 100% xylene), and mounted with cover slides with Distyrene Plasticizer Xylene. Lesions were evaluated under the microscope (Axio Imager, A1, Zeiss, Jena) (objective EC Plan-NEOFLUAR 20×/0.5, ∞/0.17), and the focus was on sensitive areas, such as the periportal spaces in the liver and the renal tubules and the glomeruli in the kidneys.

Potential Global Effects on Amphibians

To quantify the potential spatial overlap of ants and amphibians at a global scale, we obtained 1407 geographic records on *L. humile* locations from the GBIF

(Global Biodiversity Information Facility, https://www.gbif.org), AntWeb (2018) (https://www.antweb.org) and GLAD (http://globalants.org/) websites. Of 1407 *L. humile* locations, 61 were in its native range, whereas the rest were invaded locations. Amphibian ranges and IUCN status were obtained from the IUCN Red List (2017). We used the function gContains in the R package rgeos (Bivand & Rundel 2017) to extract amphibian species whose distribution polygons overlapped with the ranges of any given ant population. We then filtered this list of species by using IUCN habitat categories to exclude amphibian species that did not use macrohabitats similar to those of *L. humile* (Supporting Information).

Ants and amphibians may further be segregated by differences in microhabitat use. We used the eight categories of microhabitat, described in Moen and Wiens (2017), that adults use outside of the breeding period and included species from our dataset (Supporting Information) based on habitat descriptions from the IUCN Red List and the AmphibiaWeb database (www.amphibiaweb.org). We excluded amphibian species that only occur in aquatic, semiaquatic, or torrential microhabitats, where *L. humile* is not likely occur.

Juvenile amphibians likely use slightly different microhabitats than adults (Wells 2010; Duellman & Trueb 1994). We therefore considered the full dataset to be the maximum number of possible amphibian species overlapping spatially with the ants and the microhabitat-filtered list to be the minimum. We acknowledge that we may have overestimated risk, which is not solely determined by spatial overlap. The ant's impact will depend on the amphibian species' biological traits, such as anatomy, behavior, and physiology.

From the full data set, we determined amphibian species richness per ant locality. Then, using both the full and microhabitat-filtered data sets, we summarized cumulative species richness for amphibians co-occurring with ant populations per continent and section of continent. Finally, for each of these regions and for both datasets, we assessed the proportion of amphibian species in the 5 different IUCN Red List risk categories.

Statistical Analyses

We assumed that paralysis (in the lab or field) is equivalent to death for juveniles because it would have occurred if the juvenile remained in the Argentine ant area. We therefore analyzed the proportion of alive versus paralyzed + dead individuals with a generalized linear model with a binomial distribution and a logit link function (PROC Genmod [SAS 2008]). First, we tested whether there were differences among amphibian species and among ant species. Second, we tested the effect of the ant species within each amphibian species. In this case, we performed planned post hoc comparisons (with the *contrast* command in PROC Genmod), which compared the effects of *L. humile* with the effects of native species.

In the foraging-arena exposure experiment, we explored differences in behavior of *L. humile*, *A. senilis*, and *T.* cf. *nigerrimum* toward juvenile amphibians. Time to amphibian discovery and the maximum number of ants found on the amphibians were analyzed with generalized linear models with a gamma distribution and a Poisson distribution, respectively, and a logit link function (PROC *Genmod*, SAS 2008). Ant species and amphibian species were fixed independent variables. The number of ants in the foraging arena at the beginning of the trial and amphibian mass were covariates (the latter was only used in the model with the maximum number of ants). When the results were significant, we performed post-hoc comparisons among ant species, as explained above.

To determine differences in iridomyrmecin quantities, we used a general linear mixed-effects model (square root transformed) comparing *L. humile* and *T.* cf. *nigerrimum*; covariance within colonies was included as a random factor. The model was fitted using the lmer function in the R package lme4 (Bates et al. 2015).

The effect of toxic doses on amphibians (affected vs. unaffected) was analyzed using generalized linear models with a binomial distribution and a logit link function (glm function in the R package stats) (R Core Team 2016). Ant number per gram of amphibian, ant species, and amphibian species were the independent variables. The toxic dose, represented by the number of ants per gram of amphibian expected to elicit a toxic effect for each ant-amphibian species pair, was calculated using the function dose.p in the R package MASS (Venables & Ripley 2002) from the dose–response curves. Because iridomyrmecin quantities can vary among sites (Choe et al. 2012), we focused on the ecological ant dose, not necessarily on the toxin dose.

Relationships between the concentration of iridomyrmecin (μg/g of juvenile) in the brain and the clinical evaluation (affected vs. unaffected) were tested using a generalized linear model with a binomial distribution and a logit function (glm function in the R package stats [R Core Team 2016[); the model took amphibian species into account. Then, we examined the relationship (lm function in the R package stats) between the concentrations of iridomyrmecin (μg/g of juvenile, log transformed) in each tissue type and the quantity of iridomyrmecin (μg/g of juvenile) applied to each juvenile, which was estimated based on the species-specific iridomyrmecin contents. We also tested whether higher doses (μg/g of juvenile, log transformed) corresponded to the presence of lesions in amphibian tissues (liver and kidney). A general linear model (PROC genmod [SAS 2008]) was used for each tissue in which the identity of the amphibian species was taken into account.

Results

Local *Linepithema humile* and Juvenile Amphibian Overlap

Newly metamorphosed *E. calamita* toadlets emerging from the temporary ponds in uninvaded areas overlapped with different species of native ants. Toadlets emerging from invaded ponds overlapped only with *L. humile*, which was the sole ant species present. This ant was much more abundant during the day compared with the abundance of native ants around uninvaded ponds (Supporting Information).

Linepithema humile Depredation of and Aggression Toward Juvenile Amphibians

Along the surveyed *L. humile* trails, we observed 46 dead *H. meridionalis* (12 in 2013, 34 in 2014); 6 dead *P. cultripes* toadlets (3 in 2013, 3 in 2018); 2 dead Iberian painted frogs (*Discoglossus galganoi*) (2018); and 1 dead Iberian parsley frog (*Pelodytes ibericus*) (2018). The ants preyed on the amphibians, which ranged from being recently dead to being entirely eaten (skeletons) (Supporting Information).

When we exposed juvenile amphibian to ants in field trails, there was a significant detrimental effect of *L. humile* on juveniles, but not of *A. senilis* or *T. nigerrimum* ($\chi^2 = 10.10$, $p = 0.006$, $n = 57$, for differences among ant species) (Fig. 1a). The effects observed (alive vs. paralyzed + dead) also significantly differed among amphibian species ($\chi^2 = 6.10$, $p = 0.013$, $n = 57$). The effects of *L. humile* differed from those of the two native ants in the case of *P. cultripes* ($\chi^2 = 10.10$, $p = 0.006$, $n = 30$; planned comparisons: $p = 0.010$ in both cases), but not in the case of *H. meridionalis* ($\chi^2 = 0.00$, $p = 1.000$, $n = 27$), in which none of the froglets was affected by the ants (they always escaped). In the *L. humile* trails, 20% of the *P. cultripes* toadlets died and a further 20% were initially paralyzed but recovered after approximately 10 min (Fig. 1a).

Linepithema humile Aggressiveness in the Foraging-Arena-Exposure Experiment

The native ant *A. senilis* discovered amphibians faster than the invasive ant *L. humile* ($\chi^2 = 27.0$, $p < 0.001$, $n = 290$; $p < 0.001$ for all contrast with *A. senilis*). Moreover, the amphibians were covered by significantly more ants of *T.* cf. *nigerrimum* than of *L. humile* (mean [SE]: 17.9 ants [1.9] vs. 13.0 ants [2.0], respectively; $\chi^2 = 177.22$, $p < 0.001$, $n = 284$; <0.018 for all contrasts with *T.* cf. *nigerrimum*). Whereas the attacks by *A. senilis* or *T.* cf. *nigerrimum* had no obvious effect, those by *L. humile* ultimately resulted in a proportion of individuals paralyzed and dead ($\chi^2 = 88.56$, $p < 0.001$, $n = 294$ for differences among ant species) (Fig 1b). The effects observed (alive vs. paralyzed + dead) were also significant among amphibian species ($\chi^2 = 14.43$, $p < 0.001$, $n = 294$). The effects of *L. humile* differed from those of the 2 native ants on *P. cultripes* and on *E. calamita* ($\chi^2 = 44.31$, $p < 0.001$, $n = 94$; $\chi^2 = 39.74$, $p < 0.001$, $n = 125$, respectively; planned comparisons: $p < 0.001$ in all cases), but not on *H. meridionalis* ($\chi^2 = 4.51$, $p = 0.105$, $n = 75$). Exposure to *L. humile* had the strongest effect on *P. cultripes*; 53% of juveniles were paralyzed, and all but one died within 48 h after the trial ($n = 30$) (Fig. 1b). For *E. calamita*, 38% of toadlets were paralyzed during exposure, but they recovered ∼10 min later, and only one died ($n = 45$) (Fig. 1b). Finally, *H. meridionalis* was the least affected; only 8% of froglets were paralyzed, all of which recovered within ∼10 min ($n = 25$) (Fig. 1b).

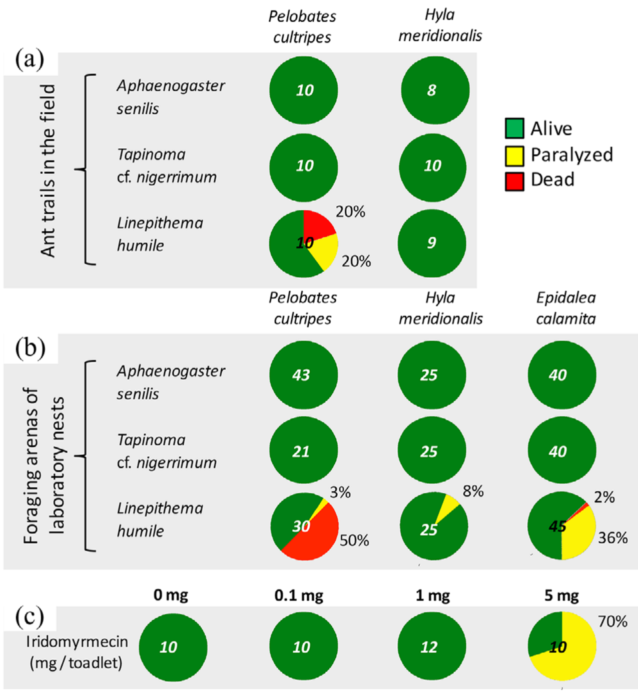
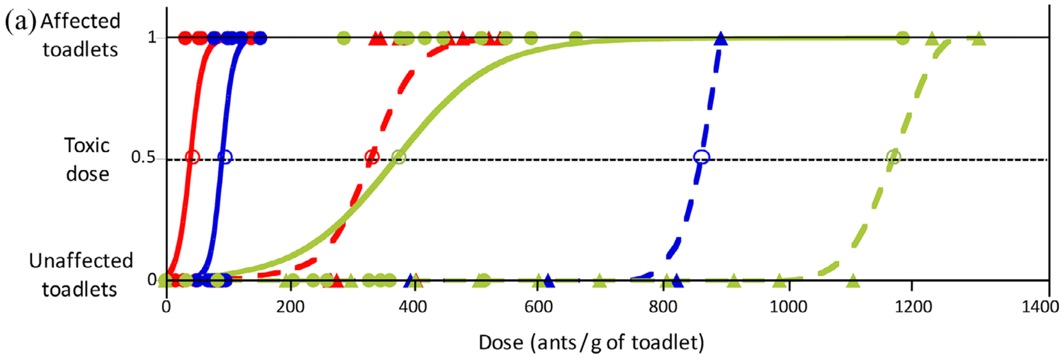


Figure 1. Effects of L. humile *on (a) juveniles of 2 amphibian species that spent 2–10 minutes in contact with ants on their trails in the field, (b) juveniles of 3 amphibian species that spent up to 10 minutes in contact with ants in the foraging arenas in laboratory nests, and on (c)* Pelobates cultripes *toadlets to which we applied 3 different concentrations of iridomyrmecin (0.1, 1, or 5 mg/toadlet, equivalent to mean of 8.15 [SE 1.13], 67.86 [6.78], and 307.62 [30.30]* Linepithema humile *workers/g of toadlet, respectively). Numbers within circles are sample sizes.*

Iridomyrmecin Quantities in *Linepithema humile*

L. humile and *T.* cf. *nigerrimum* workers had highly developed pygidial glands (Supporting Information). Iridomyrmecin (isomer 1) was the main compound



(b)

TOXIC DOSE	Number of ants / g toadlet		mg iridomyrmecin / g toadlet	
	L. humile	*T.* cf. *nigerrimum*	*L. humile*	*T.* cf. *nigerrimum*
Epidalea calamita	40.1 (9.5)	328.0 (29.9)	0.257 (0.061)	0.423 (0.039)
Pelobates cultripes	90.6 (6.5)	849.7	0.582 (0.042)	1.097
Hyla meridionalis	367.8 (44.5)	1158.5	2.360 (0.285)	1.496

Figure 2. (a) Number of Epidalea calamita, Pelobates cultripes, *and* Hyla meridionalis *(key to curve lines in [b]) affected (1) and unaffected (0) (normal or abnormal reactions, respectively, observed during clinical evaluation, see Methods) 10 min after application of mashes of different numbers of* L. humile *(solid lines and circles) and the native ant* Tapinoma *cf.* nigerrimum *(dashed lines and triangles) and (b) mean (SE) toxic dose of ants (and equivalent amount of iridomyrmecin [ant toxin]) that elicited an effect in juvenile amphibians. Standard error is only shown when meaningful. Equivalent amounts of iridomyrmecin were calculated using species-specific contents: mean 6.416 μg (SE = 0.443) for* L. humile *and 1.291 μg (1.127) for* T. *cf.* nigerrimum.

found in *L. humile* pygidial glands. *T.* cf. *nigerrimum* workers contained isomers of the main component iridodial and smaller amounts of iridomyrmecin (isomers 1 and 2) (Supporting Information). Although *T.* cf. *nigerrimum* workers were slightly larger than *L. humile* workers, the latter contained five times more iridomyrmecin (mean [SE] = 6.416 μg [0.443] vs. 1.291 μg [1.127]; $F = 135.76$, $p < 0.0001$, $n = 100$). Iridomyrmecin was 1.4% of worker fresh body mass in *L. humile* and 0.2% in *T.* cf. *nigerrimum*.

Iridomyrmecin-Exposure Experiments and Toxic Doses

According to our quantification and assuming that the ants eject all their pygidial gland content at once, the three quantities of iridomyrmecin applied (0.1, 1, and 5 mg) are equivalent, respectively, to average doses (SE) ejected by 8.4 (1.2), 69.7 (6.4), and 307.5 (30.3) *L. humile* workers/g of juvenile. We observed significant differences among treatments ($\chi^2 = 25.63$, $p < 0.001$, $n = 42$) (Fig. 1c). The lower doses were not significantly different from the control (no treatment, $p > 0.05$), with all individuals alive at the end of the experiment. However, the highest dose was different ($p < 0.001$), causing paralysis in 70% of the juveniles.

Amphibians were increasingly affected by greater numbers of ants in a dose-dependent manner ($\chi^2 = 26.69$, $p < 0.001$, $n = 81$). However, the magnitude of the effect differed, depending on both amphibian species and ant species ($\chi^2 = 23.40$, $p < 0.001$, $n = 81$ and $\chi^2 = 22.92$, $p < 0.001$, $n = 81$, respectively) (Fig. 2a). Comparatively, smaller numbers of *L. humile* caused more dramatic negative consequences than did larger numbers of *T.* cf. *nigerrimum* (Fig. 2b).

Results of the laboratory evaluations showed that the venom of the invasive ant *L. humile* had neurological effects, specifically in the medulla oblongata, pontine nucleus, and midbrain. The venom caused general paralysis (Fig. 3a), sometimes accompanied by extraocular paralysis, loss of photopupillary and palpebral reflexes, and loss of nociception response. We also observed severe damage to the skin of juveniles that came in contact with *L. humile* and of juveniles treated with iridomyrmecin (Fig. 3b).

Neurologically affected individuals had higher levels of iridomyrmecin in their brains than unaffected individuals ($\chi^2 = 10.19$, $p = 0.001$, $n = 28$). Moreover, concentrations of iridomyrmecin in brain, liver, and kidney tissue were significantly correlated with the amount of iridomyrmecin applied (brain: $F = 17.69$, $p < 0.001$, $n = 28$; liver: $F = 14.24$, $p < 0.001$, $n = 27$; kidney: $F = 8.29$, $p = 0.008$, $n = 26$) (Fig. 3c).

The histological samples revealed liver and kidney damage, indicating the toxin's acute effects on these

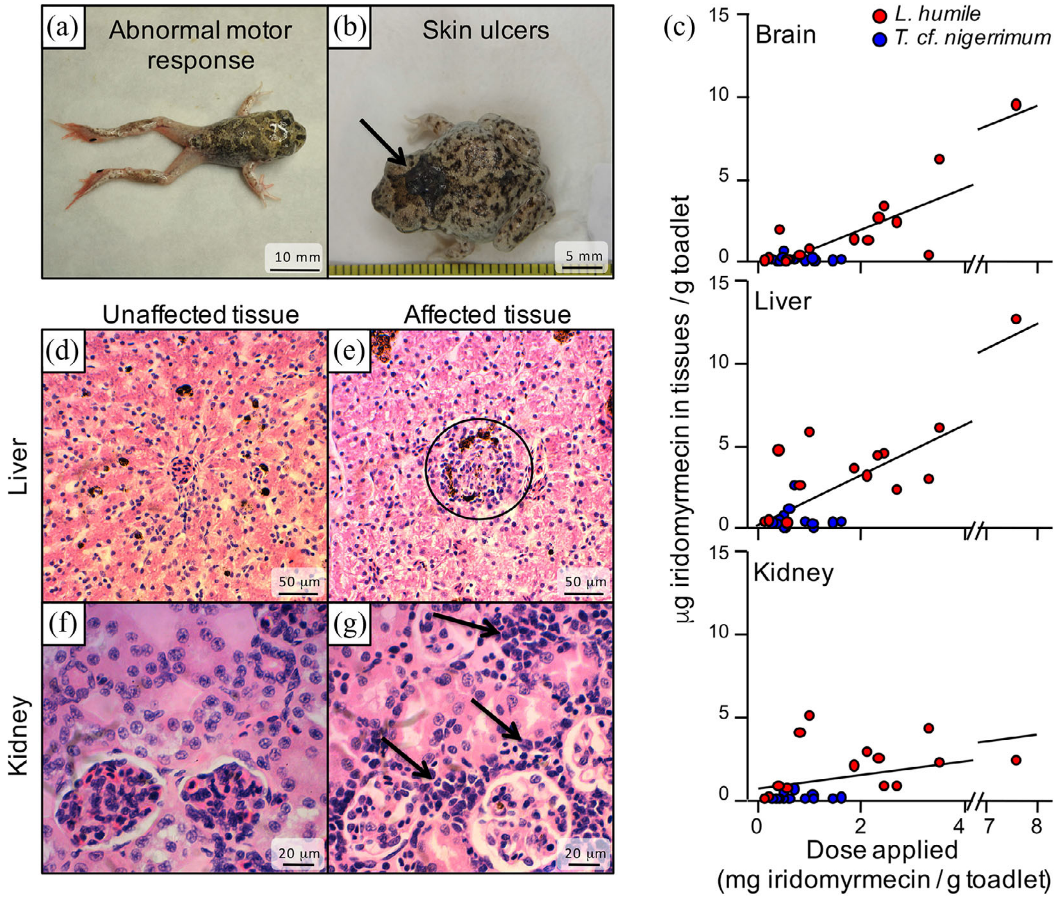


Figure 3. Pelobates cultripes *toadlet after exposure to iridomyrmecin (*Linepithema humile *toxin): (a) paralysis and (b) skin ulcers (arrow). (c) Relationship between the dose applied to toadlets (equivalent amounts of iridomyrmecin estimated from the number of* Linepithema humile *or* Tapinoma *cf.* nigerrimum *applied) and the concentration of iridomyrmecin measured in toadlet tissues after treatment (model fit determined with combined data for all amphibian species and ant species). Normal (d) liver and (f) kidney; (e) liver with lymphoplasmocytic inflammatory infiltrates in the periportal space (circled); and (g) kidney with acute tubulo-interstitial nephritis (arrows) due to exposure to iridomyrmecin.*

tissues. In the liver, we found inflammatory cell infiltrates (heterophils) around the hepatic artery (Fig. 3d, e). These lesions were observed in 16 cases ($n = 33$, all species combined). There was no significant relationship between the quantity of iridomyrmecin per gram of amphibian and the presence of lesions ($\chi^2 = 0.12$, $p = 0.727$, $n = 33$), which could be due to the individuals' short exposure to the toxin (10 min). In the kidney, we found inflammatory cell infiltrates (lymphoplasmocitary cells) in the renal tubules, which indicated tubulointerstitial nephritis (Fig. 3f, g). There were lesions in just five cases ($n = 32$, all species combined); these were found in individuals that received mean doses of 0.674, 0.665, and 1.167 mg of iridomyrmecin per gram of amphibian for *E. calamita*, *P. cultripes*, and *H. meridionalis*, respectively.

Potential Global Impacts on Amphibians

Of 1407 locations of *L. humile* populations worldwide 51 (all invasive) were not associated with any amphibian range. For the full data set, worldwide, *L. humile* populations co-occurred with 813 amphibian species (based on the 6513 terrestrial amphibian species with spatial data in the IUCN Red List database), and 9 of these amphibians exclusively co-occurred with native *L. humile* populations. Outside of its native range, *L. humile* potentially co-occurs with a mean of 11.06 (SE = 0.23) amphibian species per locality (range 1–86, $n = 1295$) (Fig. 4). When filtering the amphibian species by microhabitat, *L. humile* populations outside its native range co-occurred with 693 amphibian species (mean [SE] = 7.22 [0.20] amphibian species per locality, range 1–78, $n = 1287$).

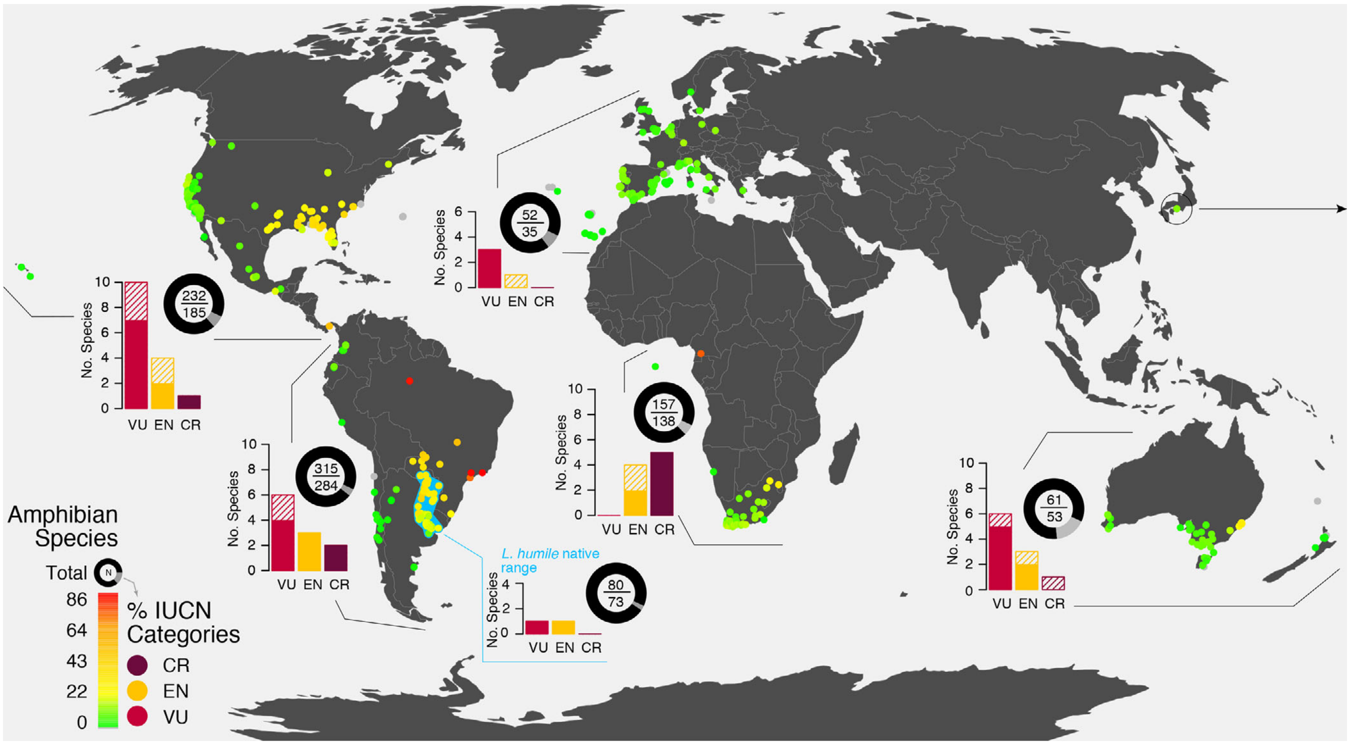


Figure 4. Records of native and invasive L. humile *populations in the regions examined and the number of co-occurring amphibian species (1-86) based on spatial and macrohabitat overlap. Pie charts show regional species richness (top number, range of cumulative number of species for the full data set; bottom number, microhabitat-filtered data set) and the proportion of unthreatened (black) and threatened (gray) species (for full data set). Bar graphs for each region show the number of vulnerable (VU), endangered (EN), and critically endangered (CR) species for the full (hashed) and microhabitat-filtered (solid) data sets.*

Discussion

We found empirical evidence that demonstrates the detrimental effect of *L. humile* ants; through their iridomyrmecin toxin, they killed juvenile terrestrial amphibians. The effect was dose and species dependent and specific to *L. humile*. Although the three tested amphibian species are listed as of least concern (*H. meridionalis* and *E. calamita*) and near threatened (*P. cultripes*) (IUCN 2017), they represent a broad phylogenetic spectrum and some of the most geographically widespread families. Worldwide, 813 amphibian species overlapped in range and macrohabitat with the Argentine ant and could therefore be affected by the species' toxin. Of these species, 6.27% are classified as threatened by IUCN (2017). At the regional level, this percentage was as high as 16.39% (Australia).

Although the most tolerant *H. meridionalis* was able to escape from the ant trails in the field soon after contact, more subtle effects were observed when the species was confined with the ants for longer periods. These findings suggest that, unlike the two other amphibian species, the jumping behavior of this frog could enable its quicker escape. Similar escape behavioral strategies have been described for juvenile *Sceloporus undulatus* lizards when encountering the red imported fire ant *S. invicta* (Langkilde et al. 2009). Moreover, juveniles of several *Hyla* species have been observed feeding on Argentine ants without any apparent negative effects (the researchers did not reported them) (Ito et al. 2009), hinting at further tolerance.

The dose-response experiments confirmed the high susceptibility of *E. calamita* and *P. cultripes* toadlets to *L. humile* attack. For example, *E. calamita* (mean mass of 0.45 g [SE 0.05] after metamorphosis) required only 20 attacking *L. humile* to result in a detrimental effect. In contrast, more than 150 workers of the native ant *T.* cf. *nigerrimum* would have been required to achieve such an effect. We attribute this difference to the larger quantities of iridomyrmecin in *L. humile* relative to *T.* cf. *nigerrimum*. Besides its greater toxicity, the augmented threat from *L. humile* arises from its high abundance and monopolization of invaded areas (e.g., around ponds) (Angulo et al. 2011; Alvarez-Blanco et al. 2017). Consequently, emerging *E. calamita* have little chance of surviving in ant-invaded areas. Moreover, this species is also especially sensitive to other drivers of global change, such as climate warming (Bosch et al. 2018).

The role of *L. humile* as a predator is not apparent and ill studied. It is mostly considered a scavenger (Angulo et al. 2011), and reports on its predation habits are scanty (Suarez et al. 2005). This is probably due to the lack of a functional sting and the ineffectiveness of its venom on humans and other mammals (Pavan & Ronchetti 1955). Moreover, it may have a delayed detrimental effect on amphibians; thus, there is no obvious association between their death and the ants.

The iridomyrmecin-exposure experiment revealed its high toxicity to amphibians, indicating that *L. humile* can cause amphibian mortality, and delineates the proximate mechanisms involved (behavioral and chemical). Understanding the mechanisms that underlie the impacts of invasive species helps scientists to assess their potential magnitude, which is essential when prioritizing and managing invasions, as is made clear in the Aichi targets of the Convention of Biological Diversity (CBD, 2020). We revealed the potential magnitude of this impact, based on the global spread of the Argentine ant (Bertelsmeier et al. 2018) in conjunction with other drivers of amphibian decline (Grant et al. 2016). We call for new research along two broad lines: determining the factors underlying venom toxicity to other amphibians (e.g., skin permeability or life-history traits, such as developmental type or breeding strategy) and examining whether the venom effect could scale to demographic effects (because population persistence is highly sensitive to the survival of juveniles in pond-breeding amphibians [Pittman et al. 2014]). This research is needed to accurately understand and contend with the worldwide impact of this invasive ant on amphibians.

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Supporting Information

Extended information on methods (Appendix S1), the functional ecology of iridomyrmecin (Appendix S2), the temporal and spatial overlap of *L. humile* ants with amphibians (Appendix S3), the identification of *L.* humile venom (Appendix S4), and a list of amphibian species across the globe overlapping with L. *humile* populations (Appendix S5) are available online. The authors are solely responsible for the content and functionality of these materials. Queries (other than absence of the material) should be directed to the corresponding author. Data are available from https://digital.csic.es/handle/10261/173421.

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Supporting Information - Index

Effects of the Argentine ant venom on terrestrial amphibians

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Appendix S1 (page 2). Extended information on methods:
A. Study area and experimental individuals.
B. Methodological details for the temporal and spatial overlap analysis of *L. humile* ants with amphibians.
C. Methodological details for the ant-trail-exposure experiment.
D. Methodological details for the foraging-arena-exposure experiment
E. Chemical analysis by gas chromatography.
F. Methodological details of the dose-response experiment

Appendix S2 (page 6). The functional ecology of iridomyrmecin. Literature review on the functional ecology of iridomyrmecin. Supporting Information Table 1. Context in which iridomyrmecin appears in previous Literature. (a) The functions for iridomyrmecin at the first mention in the text. Some studies refer to more than one function, so proportions here are referred to the total number of functions (138). (b) Main goal of the article. Data come from 116 articles expanding from 1948 to 2018. (c) Other animal taxa having and using iridomyrmecin.

Appendix S3 (page 13). The temporal and spatial overlap of *L. humile* ants with amphibians. Relative abundances over time of *Epidalea calamita* toadlets emerging from temporary ponds and **(a)** native ants or **(b)** *L. humile* ants. Invaded and uninvaded areas around ponds sampled in April May 2013 during amphibian emergence. Values represent the mean number (±SE) of toadlets per transect or ants per bait. Note the differences in axis scale between (a) and (b) regarding ants. **(c)** Mean (±SE) number of dead amphibians found along *L. humile* trails during the juvenile amphibian emergence period over three different seasons (May and June 2013, 2014, and 2018). **(d, e, f)** Examples of different phases of ant predation on amphibians: **(d)** ants attack P. *cultripes* toadlet; **(e)** freshly killed *H. meridionalis* covered by *L. humile*, around two hours after an attack; **(f)** skeleton of an *H. meridionalis* froglet, fewer than 12 h after an attack. Photo credits: Fernando Amor (d) and Elena Angulo (e,f).

Appendix S4 (page 14). The identification of *L. humile* venom. Longitudinal section of the abdomen of **a**, *Linepithema humile* and **b**, *Tapinoma cf. nigerrimum*. Partial chromatograms showing the iridodial/dolichodial iridomyrmecin complex of the pygidial glands of: **c**, *Linepithema humile* workers and **d**, *Tapinoma cf. nigerrimum* workers. **e**, List of compounds associated with the peaks in c and d. Iridomyrmecin and iridodials with different numbers are isomers. Note that the hydrocarbons may have originated from the cuticular intima lining the gland.

Supporting Information - Appendix S1

A. Study area and experimental individuals

A.1. Study area. The Doñana Biological Reserve (RBD, Spain, 36°59.491'N, 6°26.999'W) contains more than 1,100 temporary ponds, constituting the breeding grounds of eight amphibian species (Díaz-Paniagua et al., 2010). Juveniles of the various species emerge from these temporary ponds over a period of two to three weeks—in the spring for natterjack toads (*Epidalea calamita*) and in late spring or summer for Mediterranean treefrogs (*Hyla meridionalis*) and western spadefoot toads (*Pelobates cultripes*). In the 1970s, *Linepithema humile* was unintentionally introduced into the study area. Subsequently, it spread to occupy the natural habitats that surround temporary ponds, representing a patchy distribution. It has displaced native ants and established high-density colonies (Angulo *et al*., 2011).

A.2. Ethical issues. The experimental procedures were approved by the CSIC Ethical Committee and the regional government of Andalucía (CEBA-EBD 11-36, CEBA-EBD 11-36b, CSD2008-00040, 1043/MDCG/mect, 014-1073-00000613-FQH/MDCG/mect) and comply with Spanish legislation regarding the protection of wildlife used for scientific purposes. Some experiments were carried out at the Doñana Biological Station (EBD) in Seville, while others were performed at RBD. A B-M was the veterinarian in charge of animal health and welfare for the EBD and RBD experimental facilities. C D-P, P A-B, and E A were authorized to carry out animal experimentation by the Spanish MAGRAMA.

A.3. Housing of experimental animals. Juvenile amphibians were assigned to four different experiments: the ant-trail-exposure experiment was carried out in the field at RBD; the foraging-arena-exposure experiment was carried out in experimental facilities at RBD, under temperature and photoperiod conditions similar to those in the field; the iridomyrmecin-exposure and the dose-response experiments were carried out in the experimental facilities at EBD, under controlled conditions (23ºC, 12:12 photoperiod, 60% humidity). In the first two experiments, juveniles were released back near their ponds of origin 48 h after the tests. In the last two experiments, juveniles were euthanized using an overdose of anaesthetic (5-min bath in tricaine methasulfonate [MS-222], 10 g/L dissolved in Ringer's lactate solution). In the iridomyrmecin-exposure experiment, euthanasia took place 48 h after the test. In the dose-response experiment, it took place approximately 10 min after dose application, immediately after the clinical evaluation.

We collected juvenile amphibians in the field near ponds shortly after emergence. We also collected tadpoles that were laboratory-raised until reaching metamorphosis. All specimens were kept in an experimental facility, either at RBD (raised in 55-L tanks, fed common aquatic plants, under ambient temperature and photoperiod) or EBD (raised in 5-L plastic containers, fed rabbit chow *ad libitum*, 23ºC, 12:12 photoperiod). Juveniles were housed in groups (up to 10 individuals from the same pond of origin) in 20 x 30 x 20 cm terraria (with sandy substrate, pieces of cork as shelter, and a water container [in the case of *H. meridionalis*]), that were cleaned weekly. Every two days, we checked on the juveniles, misted the terraria with water, and provided individuals *ad libitum* with mealworms, *Drosophila* flies and small crickets dusted with a calcium supplement. During the experimental trials, juveniles were maintained individually in smaller containers.

A. 4. Sampling sizes. Each individual was used only once. Sampling/capture order determined the allocation of individuals to experimental groups: each new individual was assigned to a treatment on a rotating basis (i.e., treatments were alternated). Individuals were identified with a code; researchers were thus blind to treatment assignments when conducting analyses (i.e., histological, chemical analysis, clinical evaluation); behavioral tests were difficult to do blinded, especially in ant trails and foraging arenas where individuals could be at risk, or in the iridomyrmecin test in which the response occurred immediately after the administration, at the highest doses. However, it was blind in all the cases during the 48h of observations, that followed the behavioral tests. Because these were novel experiments, we had no estimates of variation for the dependent variables (i.e., the effect of the Argentine ant on juvenile amphibians), which prevented us from using power analysis to calculate a minimum sample size. Consequently, sample size was chosen so as to comply with ethical guidelines—we sought to limit the number of individuals used while ensuring that we had adequate statistical power given the numbers and types of variables in each planned analysis. In some cases, sample sizes were unbalanced due to variation in availability of amphibian species in the field.

The total number of individuals used was as follows: 185 *P. cultripes* (30 for the ant-trail-exposure experiment, 94 for the foraging-arena-exposure experiment, 42 for the iridomyrmecin-exposure experiment, and 19 for the dose-response experiment); 137 *H. meridionalis* (27 for the ant-trail-exposure experiment, 75 for the foraging-arena-exposure experiment, and 35 for the dose-response experiment); and 152 *E. calamita* (125 for the foraging-arena-exposure experiment and 27 for the dose-response experiment).

<u>A.5. Ant species.</u> Two native ant species, commonly found in RBD, *Tapinoma* cf. *nigerrimum* and *Aphaenogaster senilis*, were used for comparisons with *L. humile*. *Tapinoma* cf. *nigerrimum* is a dolichoderine ant that is closely related to *L. humile*, with whom it shares many life-history traits (Arnan et al., 2012). *Aphaenogaster senilis* is a myrmecine ant, and served as a control for the two dolichoderine ants. Five colony fragments of *L. humile*, *T.* cf. *nigerrimum*, and *A. senilis* were maintained at RBD for the foraging-arena-exposure experiment. They were housed in dark, enclosed nesting boxes (10 cm in diameter; height of 10 cm for *A. senilis* and 5 cm for *T.* cf. *nigerrimum* and *L. humile*). Each nesting box was connected to an open foraging arena (30 x 10 x 10 cm), equipped with a small Petri dish where food was permanently supplied. Another five fragments of *L. humile* and *T.* cf. *nigerrimum* colonies were maintained at EBD. They were housed in open containers (30 x 10 x 10 cm) with a dark-colored tube functioning as a nest. These fragments were used for the dose-response experiment and to carry out chemical comparisons between the ant species. All ants were fed *ad libitum* fresh fruit, mealworms, and diluted honey.

B. Methodological details for the temporal and spatial overlap analysis of *L. humile* ants with amphibians

In April and May of 2013, during the period when newly metamorphosed *E. calamita* emergence from ponds, we established two plots that were separated by 400 m. One encompassed two invaded ponds (~15 and 25 m long, respectively), and the other comprised one uninvaded pond (53 m long). The transects for ant baiting and amphibian survey were carried out at the same locations to assess spatial overlap between ants (native or invasive) and amphibians. We recorded the number and species of ants and toadlets during each sampling session. Data on ant activity can be collected using a variety of standardized methods, such as the use of baits (Savolainen et al.1988; Cerdá et al. 1997; Sanders & Gordon 2003). In this case diluted honey in water together with biscuits were used. Ants were identified by eye, and when necessary, a sample in alcohol was taking without disturbing the ants at the bait, to confirm the identity in the laboratory.

We wanted to demonstrate the directionality of interactions between ants and amphibians, i.e., do amphibians eat ants or are ants aggressive towards amphibian. Given that ants are relatively sessile organisms (relative to their nest), interactions would occur during foraging. This is why we searched for ant trails near the ponds where amphibian emerge. The amphibian would likely interact with ants when dispersing from the pond. But during preliminary observations we observed that amphibian were dying in the Argentine ant trail. Thus, we focused our sampling in counting dead juveniles.

C. Methodological details for the ant-trail-exposure experiment

We searched for at least six trails of each ant species in the field; in the case of *A. senilis,* trails were induced and maintained using bait as described in Cerdá et al. (2009). The experiment was carried out during 20 days in June, mornings or evenings, when the ants were active. We only used two amphibian species because juveniles of *B. calamita* were not available at this time of the year. We carefully positioned juveniles of *P. cultripes* and *H. meridionalis* 3 cm away from trails of the three ant species. Each amphibian was kept in place using an inverted plastic Petri dish (5.5 cm in diameter, 1.4 cm in height), enabling it to move and turn around but not to escape. The sides of the dish were perforated with eight to ten holes large enough to allow ants (either *L. humile* or *T. nigerrimum*) to enter. For the larger *A. senilis* tests we used cages (8 x 8.5 x 3 cm, with a mesh width of 5 x 5 mm). The dish or cage was held in place by hand, preventing any disturbance to the ant trail. The ants took time to discover the amphibians. Following initial contact with the ants, the amphibians were kept in place for 2 additional minutes and then released (the dish/cage was carefully removed). They were observed for up to 10 min thereafter, or until they moved at least 1 m away from the trail, whichever came first. Then they were observed for an additional 48 h in the laboratory to evaluate the effects. No amphibian died during the 10-min trials.

D. Methodological details for the foraging-arena-exposure experiment

Five artificial colony fragments (as described below) were used per ant species. Each colony received an average of six different juveniles of each of the three amphibian species, but only one juvenile per day. This frequency of exposure to the ants realistically mimic in field situation during juvenile emergence from ponds. Behavioral tests were done in the afternoon, when both ants and juvenile amphibian were active.

E. Chemical analysis by gas chromatography

For qualitative analyses of pygidial glands secretion, the glands were carefully dissected out and immersed in hexane for content extraction for at least 24h. For compound quantifications, whole ants were used rather than dissected gland to avoid possible spillage during dissection. Decyl-alcohol (99%) was used as an internal standard. The samples were run by GC/MS (Agilent) using an HP-5MS capillary column, temperature programmed from 60ºC (1 min hold) to 320ºC at a rate of 10ºC min^{-1}. Compound identification was done from the fragmentation pattern as compared to synthetic compounds.

Iridomyrmecin quantification was performed by gas chromatography (GC-FID - Shimadzu 2010 equipped with a 30 m x 0.25 mm i.d.-BPX5, 0.25 mm capillary column). Helium was used as the carrier gas (flow rate of 35.1 $ml.min^{-1}$). The injection port and detector temperatures were set to 280ºC and 310ºC, respectively. The GC oven was temperature programmed from 60ºC with a 1-min initial hold to 300ºC at a rate of $10ºC.min^{-1}$, and a final hold of 20-min. Decyl-alcohol (99%) was used as an internal standard, and the calibration curve for quantifying iridomyrmecin concentrations in the samples was constructed using synthetic iridomyrmecin (Chauhan & Schmidt 2014). The quantity of iridomyrmecin was determined by calculating the area under the peak relative to the internal standard for the different samples and corrected by the calibration curve.

To assess the percentage of iridomyrmecin of a worker 's fresh body weight, we sampled 10 ants from five laboratory colonies (used in the foraging-arena experiment) of each ant species and weighed them (in groups of 10) to obtain species-specific mean fresh weight.

F. Methodology of the dose-response experiments

Each amphibian received a single dose of mashed-ant solution (obtained from a known number of either *L. humile* or *T.* cf. *nigerrimum* workers) and was clinically evaluated 10 min later. The dose assigned for each test depended on the effects observed in previously tested individuals, to be higher or lower respectively. Doses were also adjusted according to the weight of the amphibian tested (number of ants/g of juvenile) and calculated in order to fill in the gaps in the dose-response curve. For ethical reasons, a minimal number of amphibians was used, and ant dosage levels were limited to what was necessary to obtain adequate dose-response curves (11 and 16 *E. calamita*, 14 and 5 *P. cultripes*, and 21 and 14 *H. meridionales* for the *L. humile* and the *T.* cf. *nigerrimum* curve respectively).

After the 10 min exposure to the ant doses, we performed a clinical evaluation of each individual and classified them as affected or unaffected, based on the presence (or absence) of neurological damage (Kahn 2005), including: 1) Motor response (we extended and released a leg and noted whether retraction occurred) and nociception response (presence/absence of reaction to pain inflicted by pressing a toe with tweezers), which reflected effects on the spinal cord; 2) Photopupillary reflexes (presence/absence of response to light changes) and ocular motility (ability to follow a light with the eyes), which reflected the midbrain response (i.e., in the ocular [II] and oculomotor [III] cranial nerves); and 3) The palpebral reflexes (whether the eyelid closed when we touched the medial and lateral canthus of the eye), which reflected the response of the medulla oblongata and the pontine nucleus (i.e., in the trigeminal [V] and facial [VII] cranial nerves) .

Sample preparation of amphibian tissues for lesion examination through histological analysis was carried out in the Unit of Histology of the Andalusian Molecular Biology and Regenerative Medicine Centre (https://www.cabimer.es/web3/unidades-apoyo/histologia/), following the methods described in Rojas et al. (2005).

Literature cited

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Arnan X, Cerdá X, Retana J. 2012. Distinctive life traits and distribution along environmental gradients of dominant and subordinate Mediterranean ant species. Oecologia **170**:489-500.

Díaz-Paniagua C, Fernández-Zamudio R, Florencio M, Gracía-Murillo P, Gómez-Rodríguez C, Portheault A, Serrano L, Siljeström P. 2010 Temporay ponds from Doñana National Park: a system of natural habitats for the preservation of aquatic flora and fauna. Limnetica **29**:41-58.

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Cerdá X, Retana J, Cros S 1997 Thermal disruption of transitive hierarchies in Mediterranean ant communities. Journal of Animal Ecology **66**:363-374.

Chauhan KR, Schmidt W 2014 Biorational synthesis of iridomyrmecin diastereomers from catnip oil. Tetrahedron Letters **55**:2534-2536.

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Rojas A, De Val S, Heidt AB, Xu SM, Bristow J, Black BL. 2005. Gata4 expression in lateral mesoderm is downstream of BMP4 and is activated directly by Forkhead and GATA transcription factors through a distal enhancer element. Development, **132**:3405-3417.

Sanders NJ, Gordon DM 2003 Resource-dependent interactions and the organization of desert ant communities. Ecology **84**:1024-1031.

Savolainen R, Vepsäläinen K 1988 A competition hierarchy among boreal ants: impact on resource partitioning and community structure. Oikos **51**:135-155.

Supporting Information - Appendix S2

Literature review on the functional ecology of iridomyrmecin

We searched the ISI Web of Science for the word "iridomyrmec*" to obtain published articles about iridomyrmecin (accessed 15 November 2018). The search returned 61 articles. We increased this total by finding additional articles cited therein. In each publication, at the first mention of iridomyrmecin, we noted the function of iridomyrmecin as assessed by the authors. We established the following categories for these functions: defense, insecticide, antibiotic, alarm, antibacterial, trail pheromone, cat-attracting chemical, necrophoresis, or no function specified. Publications could fall into more than one category. We also categorized each article with respect to its main subject: synthesis of iridomyrmecin, iridomyrmecin in other species, chemical composition of exocrine secretions, chemical structure, insecticide, trail pheromone, defensive compound, pharmacological research, antibiotic, necrophoresis, or alarm pheromones (Supporting Information Table S1). Finally, we analyzed (i) the relative importance of each described function of iridomyrmecin in the literature and (ii) which other species have and use iridomyrmecin and for what purpose.

The review unearthed 116 articles published between 1948 and 2018. When iridomyrmecin was assigned a function at its first mention in the text (N = 93), the two most frequently cited functions were "defense" and "insecticide" (See Table 1a, below). Most of the ant species with iridomyrmecin belong to the Dolichoderine family and notably the genera *Iridomyrmex*, *Tapinoma*, and *Dolichoderus* (See Table 1c, below). However, not all species in these genera have iridomyrmecin (e.g. *T. melanocephalum*, Tomalski et al 1987). Iridomyrmecin has also been found in non-Dolichoderinae ants (i.e. *Pheidole biconstricta*, Davidson et al. 2005), in non-ant insects, (i.e. parasitic wasps and anthicide beetles, See Table S1c, below) and in plants (Riddick et al. 2008). In all cases, iridomyrmecin has been reported to be an effective repellent. However, while Pavan and Ronchetti (1955) found that iridomyrmecin has insecticidal and antibiotic properties, they did not show that this compound was toxic for vertebrates (i.e., tests performed with dogs, rodents, and humans).

Supporting Information Table 1. Context in which iridomyrmecin appears in previous Literature. **(a)** The functions for iridomyrmecin at the first mention in the text. Some studies refer to more than one function, so proportions here are referred to the total number of functions (138). (**b**) Main goal of the article. Data come from 116 articles expanding from 1948 to 2018. (**c**) Other animal taxa having and using iridomyrmecin.

a. Function of iridomyrmecin	abbreviation (a)	%
No function specified	NS	33
Defense	DEF	22
Insecticide	INS	22
Antibiotic	ANT	7
Antibacterial	AntB	6
Alarm	AL	4
Trail	TR	3
Cat attracting chemical	CA	3
Necrophoresis	NE	1

b. Main goal of the article	abbreviation (b)	%
Synthesis of iridomyrmecin	SYN	28
Iridomyrmecin in other species	OtSp	15
Chemical composition of exocrine secretions	ExS	14
Chemical structure	CH	13
Defensive compound	DEF	9
Trail pheromone	TR	6
Pharmacologic research	PH	5
Insecticide	INS	5
Antibiotic	ANT	4
Necrophoresis	NE	1
Alarm pheromones	AL	1

c. Iridomyrmecin in other animal taxa			abbr. (c)
Ant	Subfamily Dolichoderinae	*Conomyrma* sp.	Cono
		Dolichoderus scabridus	Dsca
		Iridomyrmex nitidiceps	Inip
		Iridomyrmex pruinosus	Ipru
		Iridomyrmex purpureus	Ipur
		Tapinoma erraticum	Terr
		Tapinoma cf. *nigerrimum*	Tnig
		Tapinoma sessile	Tsess
		Tapinoma simrothi	Tsim
	Subfamily Myrmicinae	*Pheidole biconstricta*	Pbic
Non-ant	Athicid beetle	*Formicomus pedestris*	Fped
		Formicomus rubricollis	Frub
		Microhoria terminate	Mter
	Parasitic wasp	*Alloxysta brevis*	Abre
		Alloxysta victrix	Avic
		Aphidius uzbekistanicus	Auzb
		Leptopilina heterotoma	(Lhet)

Year	Reference	a	b	c
2018	Pfeiffer, L; Ruther, J; Hofferberth, J;Stökl, J. Interference of chemical defence and sexual communication can shape the evolution of chemical signals. Scientific Reports 8 (321)	DEF	OtSp	Lhet
2018	Welzel, KF; Lee, SH; Dossey, AT; Chauhan, KR; Choe, D-H. Verification of Argentine ant defensive compounds and their behavioral effects on heterospecific competitors and conspecific nestmates. Scientific Reports 8 (1477)	DEF	DEF	
2017	Bol, S; Caspers, J; Buckingham, L; Anderson-Shelton, GD; Ridgway, C; Buffington, CAT; Schulz, S; Bunnik, EM. Responsiveness of cats (Felidae) to silver vine (*Actinidia polygama*), Tatarian honeysuckle (*Lonicera tatarica*), valerian (*Valeriana officinalis*) and catnip (*Nepeta cataria*). BMC Vet Res 13:1–15	CA	OtSp	
2016	Adachi, M; Miyazawa, Y; Nishikawa, T. Improved Syntheses of (+)-Iridomyrmecin and (-)-Isoiridomyrmecin, Major Components of Matatabilactone. Nat Product Commun 11:883–886	CA	SYN	
2016	Lin, L; Cheng, XL; Li, MZ; Wang, T; Dong, MH; Wang, ZY; Liao, M. Antitumor effects of iridomyrmecin in HeLa cervical cancer cells are mediated via apoptosis induction, loss of mitochondrial membrane potential, cell cycle arrest and down-regulation of PI3K/Akt and up-regulation of IncRNA CCAT2 expression. Bangladesh J Pharmacol 11:856–862	NS	PH	
2016	Rehova, L; Dracinsky, M; Jahn, U. A general approach to iridoids by applying a new Julia olefination and a tandem anion-radical-carbocation crossover reaction. Org Biomol Chem 14:9612–9621	CA	OtSp	
2016	Scaffidi, A; Algar, D; Bohman, B; Ghisalberti, EL; Flematti, G. Identification of the Cat Attractants Isodihydronepetalactone and Isoiridomyrmecin from *Acalypha indica.* Aust J Chem 69:169–173	CA	OtSp	
2016	Stökl, J; Herzner, G. Morphology and ultrastructure of the allomone and sex-pheromone producing mandibular gland of the parasitoid wasp *Leptopilina heterotoma* (Hymenoptera: Figitidae). Arthropod Struct Dev 45:333–340	DEF	OtSp	Lhet
2015	Ebrahim, SAM; Dweck, HKM; Stokl, J; Hofferberth, JE; Trona, F; Weniger, K; Rybak, J; Seki, Y; Stensmyr, MC; Sachse, S; Hansson, BS; Knaden, M. *Drosophila* Avoids Parasitoids by Sensing Their Semiochemicals via a Dedicated Olfactory Circuit. PLoS Biol 13: e1002318	DEF	OtSp	Lhet
2015	Neff, RR. Identification and characterization of trail pheromones and queen pheromones in the Argentine ant, *Linepithema humile*. PhD thesis - University of California - Riverside, 164 pp	NS	TR	
2015	Stökl, J; Machacek, Z; Ruther, J. Behavioural flexibility of the chemical defence in the parasitoid wasp *Leptopilina heterotoma*. Sci Nat-Heidelberg 102:1–4	DEF	OtSp	Lhet
2014	Cerdá, X; van Oudenhove, L; Bernstein, C; Boulay, RR. A list and some comments about the trail pheromones of ants. Nat Product Commun 9:1115–1125	TR	TR	Tnig
2014	Chauhan, KR; Schmidt, W. Biorational synthesis of iridomyrmecin diastereomers from catnip oil. Tetrahedron Lett 55:2534–2536	DEF	SYN	
2013	Fischman, CJ; Adler, S; Hofferberth, JE. Divergent Diastereoselective Synthesis of Iridomyrmecin, Isoiridomyrmecin, Teucrimulactone, and Dolicholactone from Citronellol. J Org Chem 78:7318–7323	NS	SYN	
2013	Weiss, I; Rossler, T; Hofferberth, J; Brummer, M; Ruther, J; Stokl, J. A nonspecific defensive compound evolves into a competition avoidance cue and a female sex pheromone. . Nat Commun 4: 2767	DEF	OtSp	Lhet
2012	Choe, DH; Villafuerte, DB; Tsutsui, ND. Trail Pheromone of the Argentine Ant, *Linepithema humile* (Mayr) (Hymenoptera: Formicidae). PLoS One 7: e45016	TR	TR	
2012	Hilgraf, R; Zimmermann, N; Lehmann, L; Troger, A; Francke, W. Stereoselective synthesis of trans-fused iridoid lactones and their identification in the parasitoid wasp *Alloxysta victrix*, Part II: Iridomyrmecins in the parasitoid wasp *Alloxysta victrix*. Beilstein J Org Chem 8:1256–1264	ANT	OtSp	Avic
2012	Stökl, J; Hofferberth, J; Pritschet, M; Brummer, M; Ruther, J. Stereoselective chemical defense in the *Drosophila* parasitoid *Leptopilina heterotoma* is mediated by (-)-Iridomyrmecin and (+)-Isoiridomyrmecin. J Chem Ecol 38:331–339	DEF	OtSp	Lhet
2012	Van Oudenhove, L; Boulay R; Lenoir A; Bernstein C; Cerdá X. Substrate temperature constrains recruitment and trail following behavior in ants. J Chem Ecol 38:802–809	NS	TR	Tnig
2012	Zimmermann, N; Hilgraf, R; Lehmann, L; Ibarra, D; Francke, W. Stereoselective synthesis of trans-fused iridoid lactones and their identification in the parasitoid wasp *Alloxysta victrix*, Part I: Dihydronepetalactones. Beilstein J Org Chem 8:1246–1255	NS	SYN	Avic
2011	Martinez, MJ; Weis, EM. Field observations of two species of invasive ants, *Linepithema humile* Mayr, 1868 and *Tetramorium bicarinatum* Nylander, 1846 (Hymenoptera: Formicidae), at a suburban park in Southern California. Pan-Pac Entomol 87(1):57-61	INS	DEF	
2009	Choe, DH; Millar, JG; Rust, MK. Chemical signals associated with life inhibit necrophoresis in Argentine ants. P Natl Acad Sci USA 106:8251–8255	NE	NE	

2008	Morgan, ED. Chemical sorcery for sociality: Exocrine secretions of ants (Hymenoptera: Formicidae). Myrmecol News 11:79–90	ANT	ExS	
2008	Riddick, EW; Brown, AE; Chauhan, KR. Harmonia axyridis adults avoid catnip and grapefruit-derived terpenoids in laboratory bioassays. B Insectol 61:81–90	DEF	OtSp	
2007	Lu, Y; Zhao, YP; Wang, ZC; Chen, SY; Fu, CX. Composition and antimicrobial activity of the essential oil of *Actinidia macrosperma* from China. Nat Prod Res 21:227–233	NS	OtSp	
2006	Chang, MY; Hsu, RT; Lin, CY; Chen, BF; Lin, ST; Chang, NC. Formal synthesis of (+/-)-hop ether, (+/-)-isoboonein, and (+/-)-iridomyrmecin. Heterocycles 68:271–282	AL, DEF	SYN	
2006	Schollhorn, B; Mulzer, J. Stereocontrolled formation of three contiguous stereogenic centers by free radical cyclization – Synthesis of (+)-iridomyrmecin and (-)-isoiridomyrmecin – Formal synthesis of delta-skythantine. Eur J Org Chem 2006 (4):901–908	ANT	SYN	
2006	Zhao, YP; Wang, XY; Wang, ZC; Lu, Y; Fu, CX; Chen, SY. Essential oil of *Actinidia macrosperma*, a catnip response kiwi endemic to China. J Zhejiang Univ – Sc B 7:708–712	NS	OtSp	
2005	Davidson, DW; Clark, DA; Jones, TH. Gastral exocrine products of a myrmicine ant strongly overlap pygidial gland products of Dolichoderinae. Insect Soc 52:305–308	AL, DEF	ExS	Pbic
2000	Petersen, G; Matthiesen, C; Francke, W; Wyss, U. Hyperparasitoid volatiles as possible foraging behaviour determinants in the aphid parasitoid *Aphidius uzbekistanicus* (Hymenoptera: Aphidiidae). Eur J Entomol 97:545–550	DEF	OtSp	Auzb
1999	Hodgson, DM; Gibbs, AR; Drew, MGB. Mechanism and applications of lithium amide-induced asymmetric rearrangements of 4-substituted and 4,4-disubstituted cyclopentene oxides to cyclopentenols. J Chem Soc Perk T 1 1999: 3579–3590:	NS	SYN	
1998	Billen, J; Morgan, ED. Pheromone Communication in social insects: sources and secretions. In “Pheromone Communication in Social Insects: Ants, Wasps, Bees and Termites (Vander Meer RK, Breed MD, Espelie KE, Winston ML, eds) Westview Press, Boulder, pp 3-33	AL	ExS	
1998	Horikawa, T; Norimine, Y; Tanaka, M; Sakai, K; Suemune, H. Synthesis of optically active 9ustral[3.3.0]octane skeleton using transannular reaction. Chem Pharm Bull 46:17–21	NS	SYN	
1997	Chiu, JY; Chiu, CT; Chang, NC. Total synthesis of (+/-)-patriscabrol and (+/-)-boschnialactone. J Chin Chem Soc– Taip 44:59–63	NS	SYN	
1997	Hemp, C; Dettner, K. Morphology and chemistry of mesothoracic glands in anthicid beetles (Coleoptera : Anthicidae). Entomol Gen 22:97–108	DEF	OtSp	Fges, Fped, Mter
1997	Hodgson, DM; Gibbs, AR. An enantioselective epoxide rearrangement – Claisen rearrangement approach to prostaglandins and (+)-iridomyrmecin. Synlett 1997 (6):657–658	NS	SYN	
1997	Nangia, A; Prasuna, G; Rao, PB. Synthesis of cyclopenta[c]pyran skeleton of iridoid lactones. Tetrahedron 53:14507–14545	DEF, INS, ANT	SYN	
1997	Stepanov, AV; Veselovsky, VV. Stereocontrolled synthesis of (+)- and (-)-iridomyrmecin from citronellene enantiomers. Russ Chem Bull 46:1606–1610	NS	SYN	
1996	Nangia, A; Prasuna, G. Studies on Horner-Wadsworth-Emmons reaction in base sensitive ketones: Synthesis of (-)-mitsugashiwalactone and formal synthesis of (+)-iridomyrmecin, (-)-isoiridomyrmecin and (+)-teucriumlactone. Tetrahedron 52:3435–3450	NS	SYN	
1996	Priano, M; Pavan, M. Chemical secretions of Formicidae (Hymenoptera, Formicidae). Insect Social Life 1:173–177	NS	ExS	
1995	Ohba, M; HaneishiT; Fujii, T. Syntheses of several cyclopentano-monoterpene lactones using 1,3-dioxin vinylogous ester. Chem Pharm Bull 43:26–31	NS	SYN	
1994	Lee, E; Yoon, CH. Stereoselective favorskii rearrangement of carvone chlorohydrin – expedient synthesis of (+)-dihydronepetalactone and (+)-iridomyrmecin. J Chem Soc Chem Comm 4:479–481	NS	SYN	
1994	Völkl, W; Hübner, G; Dettner, K. Interactions between *Alloxysta brevis* (Hymenoptera, Cynipoidea, Alloxystidae) and honeydew-collecting ants: How an aphid hyperparasitoid overcomes ant aggression by chemical defense. J Chem Ecol 20:2901–2915	NS	OtSp	Abre
1993	SakaiI, K. Chiral rhodium complex-catalyzed asymmetric cyclization and its application to the synthesis of natural-products. J Syn Org Chem Jpn 51:733–743	NS	SYN	
1992	Agnel, G; Owczarczyk, Z; Negishi, E. Diastereoselective zirconocene-promoted bicyclization-carbonylation of allylically methyl-substituted enynes – synthesis of (+)-iridomyrmecin. Tetrahedron Lett 33:1543–1546	NS	SYN	
1992	Kigawa, M; Tanaka, M; Mitsuhashi, H; Wakamatsu, T. Synthesis of iridolactones isolated from silver vine. Heterocycles 33:117–120	NS	SYN	
1992	Yokoyama, Y; Tsuchikura, K. Doubly allylic strain – controlled diastereoselective intramolecular Michaell addition and a synthesis of (+/-)-iridomyrmecin. Tetrahedron Lett 33(20):2823–2824	NS	SYN	
1991	Simon, T; Hefetz, A. Trail-following responses of *Tapinoma simrothi* (Formicidae, Dolichoderinae) to pygidial gland extracts. Insect Soc 38:17–	TR, AL	TR	Tsim

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1987	Tomalski, MD; Blum, MS; Jones, TH; Fales, HM; Howard, DF; Passera, L. Chemistry and functions of exocrine secretions of the ants *Tapinoma melanocephalum* and *Tapinoma erraticum.* J Chem Ecol 13:253–263	AL, DEF	ExS	Terr
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1984	Attygalle, AB; Morgan, ED. Chemicals from the glands of ants. Chem Soc Rev 13:245–278	INS	ExS	Inip, Ipru, Dsca
1984	Cavill, GWK; Robertson, PL; Brophy, JJ; Duke, RK; McDonald, J; Plant, WD. Chemical ecology of the meat ant, *Iridomyrmex purpureus* sens. Strict. Insect Biochem 14:505–513	NS	ExS	Ipur
1983	Hefetz, A; Lloyd, HA. Identification of new components from anal glands of *Tapinoma simrothi pheonicium.* J Chem Ecol 9:607–613	NS	ExS	Tsim
1982	Cavill, GWK; Robertson, PL; Brophy, JJ; Clark, RD; Orton, CJ; Plant, WD. Defensive and other secretions of the Australian cocktail ant, *Iridomyrmex nitidiceps.* Tetrahedron 38:1931–1938	DEF	DEF	Inip
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1981	Grieco, PA; Srinivasan, CV. Stereochemical consequence of the coupling of lithium dimethylcuprate with a cyclopentenyl allylic lactone - total synthesis of dl-iridomyrmecin. J Org Chem 46:2591-2593	NS	SYN	
1981	Van Vorhis Key,SE; Gaston, LK; Baker, TC. Effects of gaster extract trail concentration on the trail following behaviour of the Argentine ant, *Iridomyrmex humilis (Mayr). J Insect Physiol 27:363–370*	INS	TR	
1980	Cavill, GWK; Davies, NW; McDonald, FJ. Characterization of aggregation factors and associated compounds from the Argentine ant, *Iridomyrmex humilis.* J Chem Ecol 6:371–384	DEF	ExS	
1980	El-Naggar, LJ; Beal JL. Iridoids. A review. Journal of Natural Products 43(6):649-707	DEF	ExS	
1979	Smith, RM; Brophy, JJ; Cavill, GWK; Davies, NW. Irididials and nepetalactone in the defensive secretion of the coconut stick insects, *Graeffea crouani.* J Chem Ecol 5:727–735	NS	OtSp	
1978	Blum, MS; Hermann, HR. Venoms and venom apparatuses of the Formicidae: Dolichoderinae and Aneuretinae. In Arthropod venoms, pp 871-894. Ed Bettini Handbook of Experimental Pharmacology	INS	DEF	
1978	Yamada, Y; Sanjoh, H; Iguchi, K. Synthesis of (+/-)-iridomyrmecin from the biogenetic precursor. Chem Lett 7:1405–1406	NS	SYN	
1977	Wheeler JW, Olagbemiro T, Nash A, Blum MS. Actinidine from the defensive secretions of dolichoderine ants. J Chem Ecol 3:241–244	DEF	DEF	Cono
1976	Cavill, GWK, Houghton, E, McDonald, FJ, Williams, PJ. Isolation and characterisation of dolichodial and related compounds from the Argentine ant, *Iridomyrmex humilis.* Insect Biochem 6:483–490	DEF	ExS	
1975	Lieberburg, I; Kranz, PM; Seip, A. Bermudian ants revisited: the status and interaction of *Pheidole megacephala* and *Iridomyrmex humilis*. Ecology 56(2):473-478	INS	DEF	
1975	Matthews, RS; Whitesell, JK. Transannular cyclizations. Steroselective synthesis of cyclopentanoid monoterpenes. J Org Chem 40(22):3312-3313	INS	SYN	
1974	Cavill GWK, Houghton E. Volatile constituents of the Argentine ant, *Iridomyrmex humilis.* J Insect Physiol 20:2049–2059	DEF	ExS	
1971	Cavill, GWK; Clark, DV. Ant secretions and cantharidin. In Naturally occurring insecticides. pp. 271-305. Ed Marcel Dekker	INS	INS	
1970	Cavill, GWK. Chemistry of some insect secretions. Liversidge Research Lecturer No. 18 – School of Chemistry, The University of New South Wales, Kensington, N.S.W. 2033. Pp 5-18	INS, DEF	ExS	
1969	Blum, MS. Alarm pheromones. Annu Rev Entomol 14:57–80	AL	AL	
1968	McGurk, DJ; Frost, J; Waller, GR; Eisenbraun, EJ; Vick, K; Drew, WA; Young, J. Iridodial isomer variation in Dolichoderine ants. J Insect Physiol 14:841–845	DEF	CH	Ipru
1967	Cavill, GWK, Clark, DV. Insect venoms, attractants, and repellents – VIII. Isidihydronepetalactone. J Insect Physiol 13:131–135	DEF	ExS	
1967	Overberber, GC; Kaye, H. Optical rotatory dispersion and conformtion of some optically active epsilon-caprolactones. J Am Chem Soc 89(22):5646-5649	NS	CH	
1966	Eisner, T; Meinwald, J. Defensive secretions of Arthropods. Science 153:1341–1350	DEF	DEF	
1965	Sakan, T; Isoe, S; Hyeon, SB, Katsumura, R; Maeda, T; Wolinsky, J; Dickerson, D; Slabaugh, M; Nelson, D. The exact nature of matatabilactone and the terpenes of *Nepeta cataria*. Terahedrom Lett 6(46):4097-4102	NS	CH	

1965	Wolinsky, J; Gibson, T; Chan, D; Wolf, H. Stereospecific syntheses of iridomyrmecin and related iridolactones. Tetrahedron 21:1247–1261	DEF, INS, ANT	SYN	
1964	McConnell, JF; Mathieson, AM; Schoenborn, BP. The crystal structure of monoterpene iridomyrmecin at -150ºC. Acta Cryst 17:472–477	NS	CH	
1964	Sisido, K; Isida, T; Utimoto, K. A synthesis of iridomyrmecin. J Org Chem 29:3361–3365	NS	SYN	
1963	Falbe, J; Weitkamp, H; Korte, F. Zur synthese bicyclischer lactone vom typ des D,L-iridomyrmecins. Tetrahedron 19:1479–1482	NS	SYN	
1962	Roth, LM, Eisner, T. Chemical defences of arthropods. Annu Rev Entomol 7:107–136	DEF, INS	DEF	
1962	Korte, F; Kochen, W; Ludwig, G; Rechmeier G; Schreiber HJ; Stiasni M; Vogel J . 14C-markierte Insektizide aus der Reihe der halogenierten Kohlenwasserstoffe und des Iridomyrmecins. Angew Chem – Ger Edit 74:503	INS	INS	
1962	Korte, F; Schreiber HJ. Insektizide im Stoffwechsel, IV. Iridomyrmecin-[3-14C]. Liebigs Ann Chem 656:145–148	INS	SYN	
1962	McConnell, JF; Mathieson, AM; Schoenborn, BP. Conformation of iridomyrmecin and isoiridomyrmecin. Tetrahedron Lett 10:445–448	NS	CH	
1961	Cavill, GWK; Hinterberger, H. The Chemistry of Ants. V. Structure and Reactions of Dolichodial. Aust J Chem 14:143–149	NS	CH	
1961	Dolejs, L; Mironov, A; Sorm, F. On terpenes .121. Structure of bulnesol and stereochemistry of guaiol, nepetalinic acids and iridomyrmecins. Collect Czech Chem Comm 26:1015–1020	NS	CH	
1960	Cavill, GWK. The cyclopentanoid monoterpenes. Reviews of Pure and Applied Chemisty. 10(3):169-183	INS	ExS	
1960	Cavill, GWK; Hinterberger, H. The chemistry of ants. IV. Terpenoid constituents of some *Dolichoderus* and *Iridomyrmex* species. Aust J Chem 13:514–519	NS	CH	Dsca
1960	Dolejs, L; Mironov, A; Sorm, F. Structure of bulnesol stereochemistry of guaiol, nepetalinic acids and iridomyrmecins. Tetrahedron Lett 11:18–21	NS	CH	
1959	Clark, KJ; Fray, GI; Jaeger, RH; Robinson, R. Synthesis of D- and L- isoiridomyrmecin and related compounds. Tetrahedron 6:217–224	NS	SYN	
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1959	Korte, F; Falbe, J; Zschocke, A. Alpha-hydroxyalkyliden-lacton-umlagerung .9. Synthese des D,L-Iridomyrmecins und verwandter bicyclischer Lactone. Tetrahedron 6:201–216	INS, ANT	SYN	
1959	Sakan, T; Fujino, A; Murai, F; Busuga,n Y; Suzuui, A. The structure of Matatabilactone. Bull Chem Soc 32(10):1154-1155	NS	CH	
1959	Wilson, EO; Pavan, M. Glandular sources and specificity of some chemical releasers of social behavior in dolichoderine ants. Psyche 66:70–76	TR	TR	
1958	Clark, KJ; Fray, GI; Jaeger, RH; Robinson, R. Eine synthese des D-und L-Iso-Iridomyrmecins. Angew Chem – Ger Edit 70:704	NS	SYN	
1958	Clark, KJ; Fray, GI; Jaeger, RH; Robinson, R. Configuration of iridodial, isoiridomyrmecin and iridomyrmecin. Chem & Industry 45:1473	NS	CH	
1958	Korte, F; Falbe, J; Zschocke, A. Synthese des D,L-Iridomyrmecins und verwandter Lactone. Angew Chem – Ger Edit 70:704	NS	SYN	
1958	Pavan, M; Trave, R. Études sur les Formicidae. IV Sur le venin du Dolichodéride *T. nigerrimum.* Insect Soc 5:299–308	INS, AntB	DEF	
1958	Wendler, NL; Slates, HL. Studies in the Iridomyrmecin series - abnormal ring closure of a 1,6-keto aldehyde. J Am Chem Soc 80:3937–3939	NS	CH	
1957	Cavill, GWK; Locksley, HD. The chemistry of ants. II. Structure and configuration of iridolactone (isoiridomyrmecin). Aust J Chem 10:352–358	NS	CH	
1956	Cavill, GWK; Ford, DL; Locksley, HD. The chemistry of ants. I. Terpenoid constituents of some Australian *Iridomyrmex* species. Aust J Chem 9: 288–293	ANT, INS	ExS	
1955	Fusco, R.; Trave, R.; Vercellone, I. Ricerche sull'iridomirmecina, l'insetticida naturale secreto dalla *Iridomyrmex humilis* Mayr. La Chimica e l'Industria 37(4):251–259	INS	INS	
1955	Fusco R, Trave R, Vercellone A. La struttura dell'iridomirmecina. La Chimica e l'Industria 37:958-959	INS	CH	
1955	Pavan, M. Sull'attività fitoinibente della iridomirmecina su *Lupinus albus*. Boll Soc Ital Biol Sper 31:967–969	ANT, AntB	PH	
1955	Pavan, M; Baggini, A. Ricerche sull'attività fitoinibitrice dell'iridomirmecina su *Lupinus albus*. Boll Zool 22:393–404	INS	PH	
1955	Pavan, M; Ronchetti, G. Studi sulla morfologia esterna e anatomia interna dell' operaia di *Iridomyrmex humilis* Mayr e ricerche chimiche e biologiche sulla iridomirmecina. Atti Soc It Sc Nat 94:379–477	INS, AntB	PH	
1955	Pavan, M; Valcurone, ML. Antagonismo della iridomirmecina verso l'effetto oncogeno della colchicina e del gammaesano su *Lupinus albus*.	DEF	PH	

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1951	Pavan, M. Sull'attivita insetticida della iridomirmecina. Mem Soc Ent It 30:107–132	INS	INS
1950	Pavan, M. Potere insetticida della iridomirmecina e significato della sostanza nella biologia di *Iridomyrmex humilis* Mayr (*Formica argentina*). La Ricerca Scientifica 20:1835–1855	INS	INS
1950	Zyka, J. New antibiotics, chloromycetin, aureomycin, and iridomyrmecin. Cas Cesk Lek 63(15):172-173	INS, AntB	ANT
1949	Pavan, M. Ricerche sugli antibiotici di origine animale. Nota riassuntiva	AntB	ANT
1948	Pavan, M; Nascimbene, A. Studi sugli antibiotici di origine animale. I. Su un principio antibiotico di *Iridomyrmex pruinosus humilis* Mayr. (nota prev.). Boll Soc Med Chir Pavia 62:193–197	ANT	ANT
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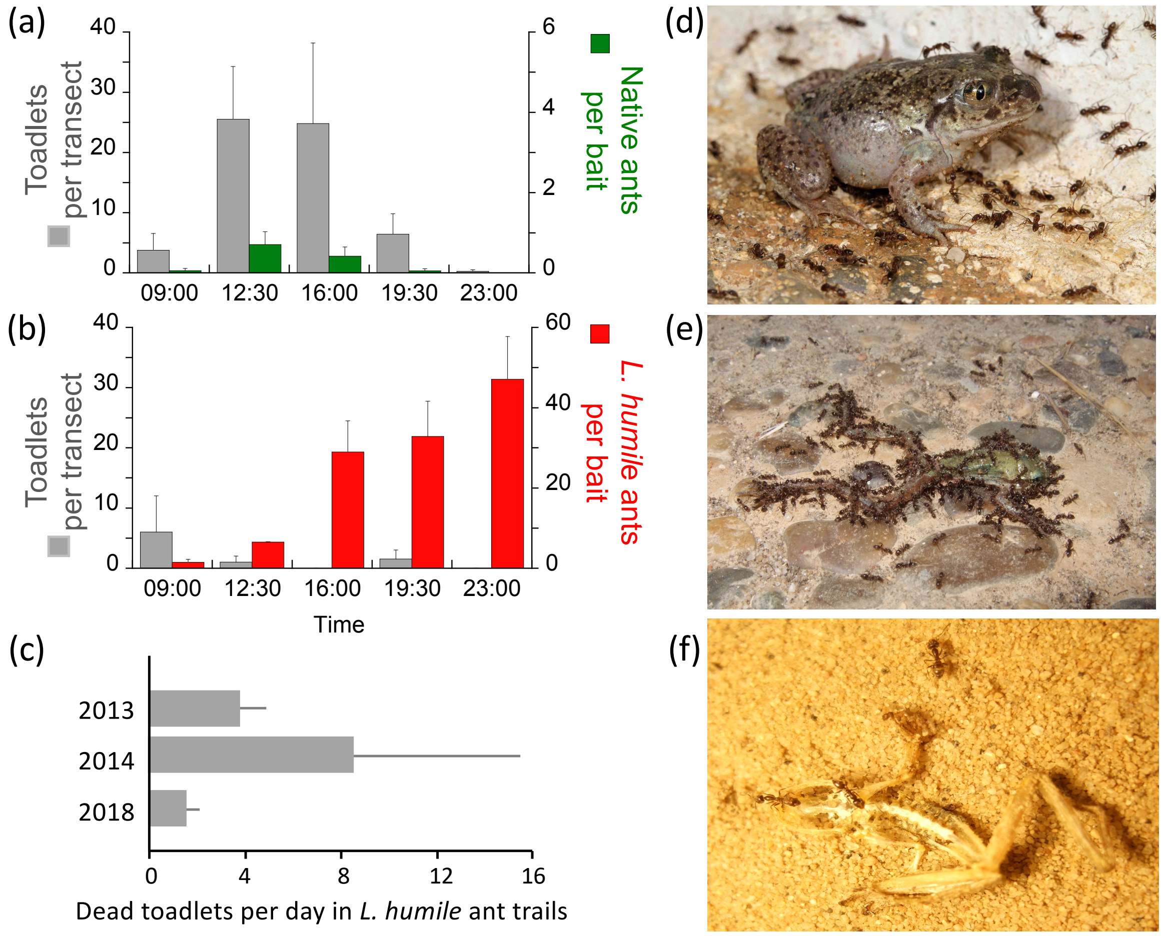
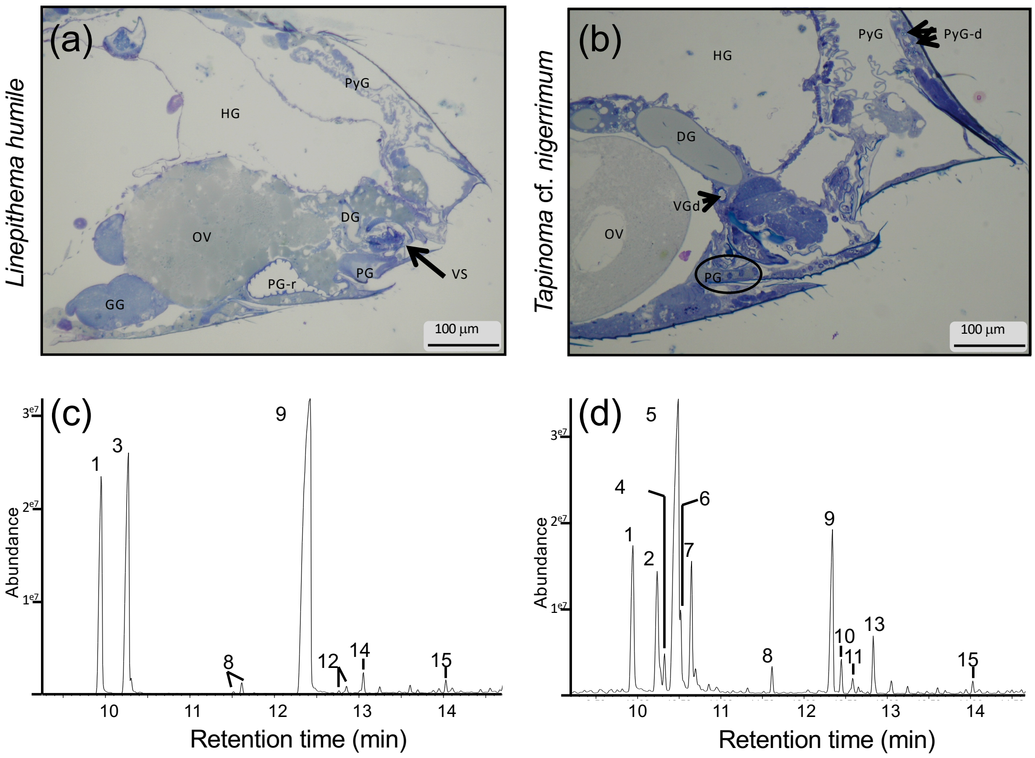


Supporting Information S3. *Linepithema humile* ants overlap temporally and spatially with amphibians Relative abundances over time of *Epidalea calamita* toadlets emerging from temporary ponds and **(a)** native ants or **(b)** *L. humile* ants. Invaded and uninvaded areas around ponds sampled in April May 2013 during amphibian emergence. Values represent the mean number (±SE) of toadlets per transect or ants per bait. Note the differences in axis scale between (a) and (b) regarding ants. **(c)** Mean (±SE) number of dead amphibians found along *L. humile* trails during the juvenile amphibian emergence period over three different seasons (May and June 2013, 2014, and 2018). **(d, e, f)** Examples of different phases of ant predation on amphibians: **(d)** ants attack *P. cultripes* toadlet; **(e)** freshly killed *H. meridionalis* covered by *L. humile*, around two hours after an attack; **(f)** skeleton of an *H. meridionalis* froglet, fewer than 12 h after an attack. Photo credits: Fernando Amor (d) and Elena Angulo (e,f).



Glands:
PyG, PyG-d: Pygidial gland and duct
PG,PG-r: Pavan gland and reservoir
VG, VGd: Venom gland and duct
DG: Dufour gland

Other structures:
GG: ganglion
HG: hindgut
OV: ovariole
VS: vestigial sting

(e)

Peak number in: (c)	(d)	Compound
1	1	1-Decanol (IS)
	2	Iridodial 1
3		Dolichodial
	4	Tridecane
	5	Iridodial 2
	6	iridodial3
	7	Iridodial 4
8	8	Tetradecane
9	9	Iridomyrmecin 1
	10	Iridomyrmecin 2
	11	Dihydronepenta lactone
12		Pentadecane
	13	2-tridecanone
14		3-methyl pentadecane
15	15	Hexadecane

Supporting Information S4. Identification of *Lineptihema humile* ant venom. Longitudinal section of the abdomen of **a,** *Linepithema humile* and **b,** *Tapinoma* cf. *nigerrimum*. Partial chromatograms showing the iridodial/dolichodial iridomyrmecin complex of the pygidial glands of: **c,** *Linepithema humile* workers and **d,** *Tapinoma* cf. *nigerrimum* workers. **e,** List of compounds associated with the peaks in **c** and **d**. Iridomyrmecin and iridodials with different numbers are isomers. Note that the hydrocarbons may have originated from the cuticular intima lining the gland.