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The costs and effectiveness of chemical defenses in herbivorous insects: a meta-analysis

Elena L. Zvereva[1] and Mikhail V. Kozlov

Section of Ecology, University of Turku, 20014 Turku, Finland

Abstract. The evolution of defensive traits and strategies depends on the intensity of selection imposed by natural enemies and on the fitness costs of defenses against these enemies. We tested several hypotheses about the evolution of chemical defenses in plant-feeding insects using a meta-analysis. We analyzed the effectiveness (in terms of prey survival; 159 publications) and costs (in terms of reduction in performance due to defense production; 33 publications) of chemical defenses in various prey–predator systems (140 herbivore species and 124 enemy species). The chemical defenses of insect herbivores, on average, were effective against generalist predators, were not effective against specialist predators and generalist parasitoids, and increased the risk of parasitism by specialist parasitoids. The defenses were more effective against vertebrate than against invertebrate predators and most effective against birds. Defensive compounds synthesized *de novo* and derived from the herbivore's food plants did not differ in the magnitude of their effects. Externalization of chemical defenses enhanced their effects on naïve vertebrate predators but simultaneously increased the risk of parasitism. The defenses of specialist herbivores were more effective than those of generalists, mostly due to species that sequestered plant allelochemicals for their own defenses. Advertising of chemical defenses by warning display enhanced their effectiveness only against vertebrate predators. Aposematic colors and patterns were more effective warning signals than other types of conspicuous coloration against both experienced and naïve vertebrate predators, suggesting that certain colors and/or patterns were more important than conspicuousness for both learning and innate avoidance. The meta-analysis did not reveal physiological costs of the production of chemical defenses across 22 herbivore species, although the results varied strongly with the method used to measure these costs. We conclude that the cost–benefit trade-offs driving the evolution of chemical defenses in herbivorous insects are affected by ecological costs (i.e., increased susceptibility to parasitoids) more than by costs in terms of resources. Still, a favorable cost–benefit ratio, i.e., great effects for a small expenditure, may partly explain the prevalence of chemical anti-predator defenses in insects.

Key words: allelochemicals; aposematism; birds; cost–benefit trade-offs; de novo *synthesis; defense externalization; diet specialization; experimental methods; meta-analysis; parasitoids; predator experience; predators; prey survival; sequestration.*

INTRODUCTION

Chemical defenses of animals against their natural enemies have always fascinated biologists. Several scientific and popular books (Blum 1981, Ruxton et al. 2004, Eisner et al. 2007, Waldbauer 2012) are devoted to this topic, and the most interesting examples of insect "chemical weapons" have attracted considerable attention from the general public. Additionally, warning displays, which are frequently found in chemically defended species and produce an immense diversity of "beautifully and artistically colored" creatures, have been the focus of especially intense and justifiable attention from biologists since the time of Wallace and Darwin (Ruxton et al. 2004). The general interest in this research field is supported by intriguing questions about how evolution may have shaped this incredible variety of defensive mechanisms and warning signals advertising these defenses to predators.

Chemical defenses are extremely taxonomically and ecologically widespread among animals, and their diversity across species and phyla is impressive (Blum 1981, Ruxton et al. 2004). Investigations of chemical defenses among plant-feeding insects are of special interest because many herbivores use various plant secondary metabolites (allelochemicals), evolved as plant antiherbivore defenses, for their own defenses against natural enemies (Duffey 1980, Nishida 2002, Opitz and Müller 2009). This ability of herbivores to sequester plant defenses for their own benefit is one of the most excit-

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[1] E-mail: elezve@utu.fi

ing examples of tri-trophic interactions in nature and is tightly linked with the evolution of host plant specialization in herbivores. In particular, generalist enemies may provide a driving force for the narrowing of host plant range in herbivores (Bernays and Graham 1988). At the same time, other herbivores produce their own toxins autogenously, i.e., by *de novo* synthesis, or by combining these two strategies. For some groups of herbivores, e.g., leaf beetles, sequestration of chemicals is a derived feature while *de novo* biosynthesis is ancestral (Pasteels et al. 1988, Fürstenberg-Hägg et al. 2014), whereas in some cyanogenic butterflies, *de novo* synthesis is considered typically as a phylogenetically derived strategy (Brown and Francini 1990). Thus, the evolutionary relationships between sequestering of defensive compounds and manufacturing them *de novo* are diverse (Engler-Chaouat and Gilbert 2007). Quantitative comparisons of costs and effectiveness between these two strategies could provide a key to understanding the evolution of chemical defenses (Nishida 2002).

A substantial body of work has been devoted to the identification of defensive compounds. To date, more than 800 individual chemicals have been reported to have defensive functions (El-Sayed 2014). The rapid development of analytical methods in the 1970s–1980s resulted in a concentration of research efforts on the chemistry of defensive compounds. However, the biological functions of these compounds frequently remained unexplored, or the repellency/deterrence of the synthesized compounds was estimated in the simplest laboratory bioassays (Blum 1981, Pasteels et al. 1983, Aldrich 1988). Still, a considerable amount of data accumulated during the decades of intensive research on both chemical and ecological aspects of prey–predator interactions, and these data have repeatedly been summarized in narrative reviews. The early reviews covered all chemical defenses of arthropods (e.g., Roth and Eisner 1962, Blum 1981, Pasteels et al. 1983). However, due to the rapid development of this field, subsequent reviews have generally had a narrower scope, being restricted to a certain mechanism of defense production, e.g., sequestration (Nishida 2002, Opitz and Müller 2009), a certain group of insects (Pasteels et al. 1988, Nishida 2002) or a certain group of defensive chemicals (Aldrich 1988, Hopkins et al. 2009, Trigo 2011). These later reviews erected and discussed a number of hypotheses on the evolution of chemical defenses, but it remains unclear whether most of these hypotheses can be generalized across a broad range of both prey and enemy species and types of chemical defenses.

The evolution of defensive traits is commonly considered in terms of trade-offs between the benefits gained from protection against enemies and the costs of these defenses (Bowers 1992, Camara 1997). Therefore, quantitative explorations of sources of variation in both effectiveness and costs of defenses have potential to enhance our understanding of the evolution of defensive strategies in insects. Such a quantitative analysis can be implemented with meta-analysis, a powerful, informative, and objective tool that offers a number of advantages over narrative reviews (Koricheva and Gurevitch 2013). Numerous meta-analyses in the field of plant antiherbivore defenses (e.g., Koricheva 2002, Koricheva et al. 2004, Kaplan et al. 2008, Barbosa et al. 2009) have considerably advanced the understanding of plant–herbivore interactions, but no such research synthesis has been attempted for antipredatory defenses in insects.

The fitness costs associated with chemical defenses are usually classified as allocation, opportunity, environmental, self-damage, and plasticity costs (Tollrian and Harvell 1999, Ruxton et al. 2004). Some of these costs may occur only in specific circumstances, and some are difficult to measure, e.g., constitutive allocation costs (Cogni et al. 2012). The most frequently studied allocation costs are physiological costs (in animals, also called metabolic or energetic costs), i.e., allocation of limited resources to production, maintenance, and operation of a defense at the expense of other fitness-related functions of an organism. These costs may be measured most obviously as trade-offs between investments in defense and in some other components of fitness, such as growth, survivorship, or fecundity (Camara 1997, Ruxton et al. 2004). The primary studies to date have provided contradictory evidence about the existence and magnitude of the physiological costs of chemical defense (reviewed in Ruxton et al. 2004), and the sources of variation in the outcomes of these studies remain unclear. In addition, some patterns have been assumed general when there is little supporting evidence. For example, physiological costs of *de novo* synthesis of defensive chemicals are frequently believed to be higher compared to costs of sequestration of plant allelochemicals (e.g., Fürstenberg-Hägg et al. 2014), and this presumption is used to explain why sequestration is widely distributed among herbivores from a variety of insect lineages (Bowers 1992, Nishida 2002). However, evidence for this difference in costs is scarce and has been demonstrated only for some leaf beetle species (Rowell-Rahier and Pasteels 1986).

The benefits of chemical defenses are more evident than their costs, because these defenses generally increase the survivorship of their possessors in the presence of natural enemies. However, none of the defensive strategies is universal, and their protective effectiveness depends on many factors, including characteristics of both prey and enemy and the ecological context in which they interact. To estimate the relative selection pressure from natural enemies on the evolution of chemical defenses, we need to compare the effectiveness of defenses against various enemies. For example, while chemical defenses increase prey survival against many types of predators, lepidopteran caterpillars containing defensive chemicals were found to be preferentially parasitized (Gentry and Dyer 2002), which may indicate that the chemical defenses of these caterpillars are not effective against parasitoids. Furthermore, the dietary specialization of both prey (Dyer and Floyd 1993) and natural enemies

(Glendinning 2007) may contribute considerably to the outcome of their encounter. The effects of defenses may also depend on the characteristics of defensive chemicals, the location of their storage, and the ability of a prey to externalize these chemicals (Pasteels et al. 1983). For example, defensive compounds are frequently classified into toxic (truly poisonous for the predator), and generally harmless volatile repellent and deterrent compounds; toxic compounds are usually held in the body of the prey, while generally harmless compounds are usually expelled from specialized glands and irritate the chemical sense receptors of predators (Pasteels et al. 1983). Although these categories are not mutually exclusive, as some chemicals can cause multiple effects, the toxins are believed to be ecologically far less effective than repellents (Brower 1984), because toxic effects are generally delayed and do not increase the individual survival of a prey. It has also been hypothesized that toxins are directed preferentially against vertebrate predators, whereas repellents are directed primarily against invertebrate predators (Pasteels et al. 1983).

Many chemically defended species advertise their unprofitability for enemies by displaying warning signals (visual or acoustic) and thus avoid enemy attack. Studies of aposematism cover various aspects of prey–predator interactions, including the effects of different characteristics of aposematic display on perception, learning, and innate wariness of predators; the roles of the background; and the presence of other prey (reviewed by Ruxton et al. 2004). However, these studies frequently use experiments with domestic chicks and artificial prey models, which can provide biased information, in particular because chicks belong to a species that has been bred for a diminished fear of new food items (Marples and Kelly 1999), and perception of artificial prey bearing a single signal may well differ from perception of the real prey possessing the same signal in combination with other features. Therefore, it is important to test whether predictions based on these experiments are confirmed in studies of natural prey–predator systems.

The extreme diversity of insect chemical defenses and massive amount of accumulated data call for quantitative research synthesis addressing natural prey–predator systems. The aims of our meta-analysis are to (1) estimate the magnitudes of physiological costs of chemical defenses (in terms of reduction in performance due to defense production) and of benefits gained by their possessors (i.e., effectiveness of defenses measured in terms of prey survivorship) across diverse natural tritrophic level systems, and (2) explore the most important sources of variation in both costs and the effectiveness of defenses related to taxonomy, morphological characteristics, and life history traits of herbivorous prey and their natural enemies, defense chemistry, defensive strategies, and methods applied in primary studies. In particular, we test the following hypotheses that have been erected in various case studies and narrative reviews: (1) chemical defenses impose physiological costs on their possessor; (2) *de novo* synthesis of defensive substances is more costly than sequestration of chemicals from food plants; (3) chemical defenses are more effective against predators than against parasitoids; (4) chemical defenses are more effective against generalist than against specialist enemies; (5) chemical defenses are more effective against experienced than against naïve predators; (6) defenses of specialized herbivores are more effective than defenses of generalist feeders; (7) externalization of defenses increases their effectiveness against enemies; and (8) aposematic signals, mechanical defenses, and defensive and gregarious behavior of prey enhance the effects of chemical defenses.

MATERIALS AND METHODS

Data collection and response variables

We searched for publications in the ISI Web of Science database using combinations of several keywords (“chemical”, “defen*”, “predat*”, “parasit*”, “cost*”, “secretion*”) and examined the reference lists of the identified case studies and of narrative reviews discussing chemical defenses of insects. The search was completed on 15 January 2015.

To be included in our meta-analysis, a study had to fit the following criteria:

1) The reported information makes it possible either to estimate physiological costs or the effectiveness of chemical defenses in herbivorous insects.
2) Physiological costs were measured as (1) changes in herbivore performance indices in response to deprivation of produced secretions (simulation of predator attack) or (2) correlations between performance indices and concentrations or amounts of sequestered defensive chemicals in the insect. Studies comparing herbivore performance indices on plants with different concentrations of allelochemicals were accepted only if concentrations of defensive compounds in insects were known to be proportional to their concentrations in the diet.
3) The effectiveness of chemical defenses was measured in field or laboratory experiments that used either a prey (usually alive) or defensive compounds obtained from a prey (i.e., experiments with chemically synthesized defensive chemicals were excluded).
4) The effectiveness of chemical defenses was measured as survival of defended prey (relative to non-defended prey) exposed to predators or as other indices associated with survival: frequency of predator attacks, prey acceptance by predator (palatability), and deterrent or repellent activity of defensive secretions or body extracts of defended prey compared to pure solvent. Effects of prey defenses on predator/parasitoid fitness and time of prey processing by predator were excluded.
5) The experimenter compared defended and non-defended (control) prey; experiments lacking controls (e.g., reporting mortality of defended prey

only or comparing the effects of defenses which have different compositions) were excluded.

6) The effects of defenses were tested against natural enemies (parasitic or predatory) to which the prey may be exposed in nature (i.e., experiments with domestic chicks and experiments using encapsulation of artificial implants as a measure of defenses against parasitoids were excluded).
7) Means accompanied by variances and sample sizes or numbers (percentages) were reported for defended and control prey, or correlation coefficients and sample sizes were reported for the association between defenses and performance indices, or this information could be obtained from the authors, or it was possible to estimate missing data from the test statistics.

We took some measures to avoid non-independence of data used in meta-analysis. When data on several similar experiments were presented within one publication, we selected the experiment with the highest number of prey items. When the study reported several subsequent records (for example if survival was measured several times), we selected the final record. When several concentrations of allelochemicals in the diet were tested, we selected the diet with the most realistic concentration (similar to concentration in the host plant) and compared it with the diet either lacking allelochemicals or containing their lowest concentration.

Classificatory variables

Natural enemies were classified by their trophic strategy (predator or parasitoid), diet breadth (specialists or generalists, according to information provided in the primary study), high-rank taxonomy (class, order), and the stimuli used to locate a prey (visual or chemical). Whenever possible, we classified predators as naïve, experienced in the experiment with a certain prey, or wild-caught or observed in nature, i.e., having uncontrolled experience with a wide range of prey (wild-caught hereafter).

Prey species were classified by their high-rank taxonomy (order), developmental stage (eggs, larvae, pupae, adults), and diet breadth (specialists: feeding on narrow range of host plants, usually sharing secondary chemistry; generalists: feeding on wide range of host plant species differing in secondary chemistry). We considered several characteristics of prey chemical defense: chemistry of a major compound, its origin (derived from host plant or synthesized *de novo*), toxic and/or deterrent properties of defensive compounds (according to information provided in primary studies or reviews), and defense externalization (absent: defensive chemicals are stored in hemolymph and body tissues; present: expelling secretions from glands, reflex bleeding, regurgitation, deposition in fecal shields). Within defenses derived from host plants, we distinguished sequestration, when plant allelochemicals are transported through the gut wall and accumulated in the body tissues or in specialized glands, and acquisition, when plant allelochemicals are used for defense without their accumulation in the body (mostly contained in regurgitant or fecal shields).

Non-chemical warning signals displayed by prey species were classified as acoustic or visual. Based on coloration, we classified prey as true aposematic (yellow, orange, and/or red coloration, usually forming a high-contrast pattern with black), other conspicuous (e.g., totally black or metallic), and non-conspicuous (cryptic and living in soil or in plant tissues), and characterized their gregariousness as truly gregarious, living in small groups, and solitary. We also classified prey species as possessing or not possessing physical defenses (hairs, spines) and as displaying or not displaying active defensive behavior not related to chemical defenses (thrashing, wriggling, whipping, arching, biting). In categorizing insects by their dietary specialization, gregariousness, physical defenses, and active defensive behavior, we generally used the information provided by the authors of primary studies. If the necessary information was missing in the case study, we searched for it in various publications.

Variables related to experimental design included the type of environment (laboratory or field), method of defense presentation to predator (natural prey or extracted defensive compounds offered on non-defended prey or in drinking water), measured variable (deterrence, prey acceptance, prey survival, frequency of predator attacks), and type of control (another [non-defended] prey species or the same prey species lacking defenses due to depleting of secretions or rearing on a diet lacking allelochemicals). When analyzing the physiological costs of defenses, we compared two main methods: (1) secretion depletion, inducing intensive production of defensive compounds; decrease in performance of such depleted insects is attributed to physiological/energetic costs (can be applied only when insects externalize the secretions), and (2) prevention of defense production in control insects by rearing them on a diet lacking precursors; decreased performance of insects producing defensive compounds compared to control insects was attributed to physiological/energetic costs (can be applied only when insects sequester/acquire defensive compounds from their food).

Meta-analysis

We used Hedges' d measure of the effect size (ES), calculated as the difference between the means of the experimental and control groups divided by the pooled standard deviation and weighted by sample size. When the data were reported as proportions, Hedge's d was obtained from the odds ratio using the online calculator (*available online*).[2] When costs of defenses were reported as correlation coefficients (r) between concentrations of defensive compounds and performance

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[2] http://www.campbellcollaboration.org/escalc/html/EffectSizeCalculator-SMD9.php

indices, we calculated Hedges' d using the equation: $d = 2r/\sqrt{(1 - r^2)}$ (Rosenberg et al. 2000). ESs for effectiveness calculated from means did not differ from ESs calculated from the odds ratio (120 and 234 ESs, respectively; $Q_B = 1.81$, df = 1, $P = 0.18$), and ESs for costs calculated from means did not differ from ESs calculated from correlations (74 and 13 ESs, respectively; $Q_B = 0.86$, df = 1, $P = 0.36$), allowing us to combine ESs calculated from different types of data in further analyses. In the analysis of defense effectiveness, we considered ES to be positive when the defenses increased survival and negative when the survival of the defended prey decreased. Thus, positive ESs indicate effective defenses, while negative ESs indicate increased susceptibility to enemies. In the analysis of physiological costs, we considered ES to be negative when the measured performance indices decreased relative to the control. Thus, a negative ES indicated that costs were present.

All analyses were performed using the random effects categorical models (that used the inverse variance of d as weights) in the MetaWin 2.0 program, assuming that studies differ not only by sampling error, but also by a random component in ESs (Rosenberg et al. 2000). The effectiveness and costs of defenses were considered to be statistically significant if the 95% confidence interval of the mean ES (CI_{95}) did not overlap zero. The variation in the ES values within and among the classes of categorical variables was explored by calculating the heterogeneity indices (Q_T and Q_B, respectively) and testing these against the χ^2 distribution (Koricheva et al. 2013).

Temporal trends in the research of defense effectiveness and costs were explored by two methods. First, we calculated Pearson correlation coefficient between publication year and ESs averaged by publication year (SAS Institute 2009). To further explore temporal trends, we conducted cumulative meta-analysis following Leimu and Koricheva (2004). Cumulative ESs and CI for all studies were calculated by study year separately for effectiveness and costs of defenses. Proportions of species possessing specific morphological, defensive, and other life history traits (among species included in our database) were compared by frequency analysis (χ^2 test; SAS Institute 2009).

Results

Databases

A total of 354 ESs were calculated from 159 papers published between 1958 and 2014 for the effectiveness of defenses, and 87 ESs were calculated from 33 papers published between 1985 and 2012 for the costs of defenses (Supplement). The magnitudes of the reported effects decreased with publication year for the effectiveness of defenses ($r = -0.57$, $n = 35$ yr, $P = 0.0004$) but not for the costs of defenses ($r = 0.19$, $n = 18$ yr, $P = 0.44$). This result is supported by cumulative meta-analysis, which revealed clear temporal changes in the magnitude of the reported effects for effectiveness of defense (Fig. 1A), while for costs of defense these changes were not evident (Fig. 1B).

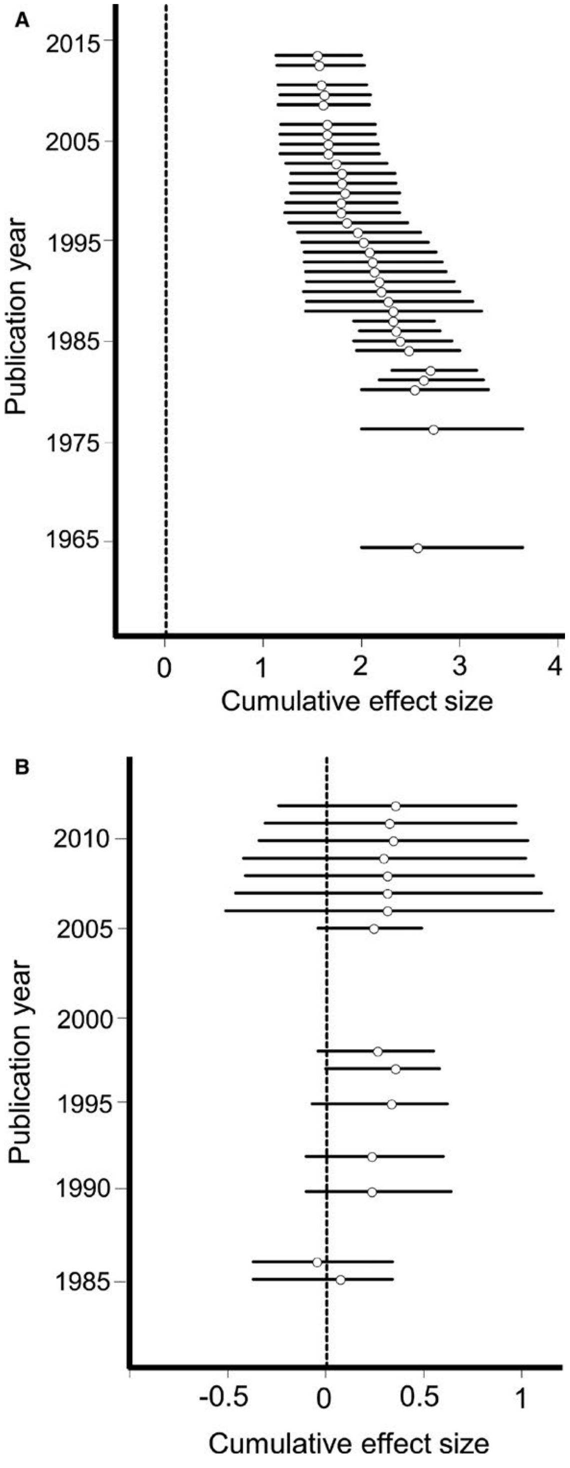

Fig. 1. Temporal trends in the magnitudes of the cumulative Hedges' d effect sizes (ESs) reflecting (A) effectiveness of herbivore chemical defenses against natural enemies, and (B) costs of these defenses. Cumulative ESs are calculated by publication year.

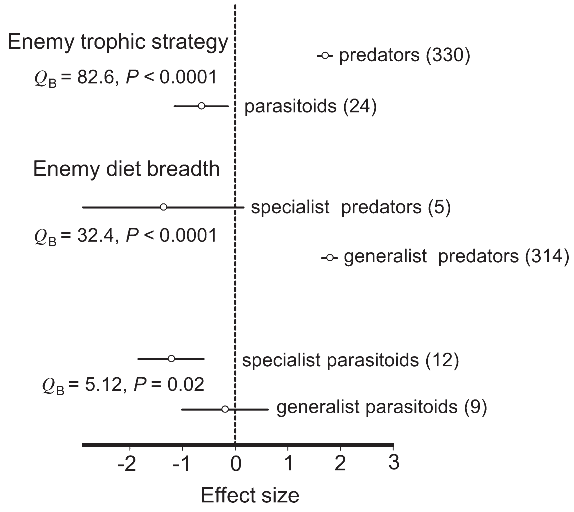

FIG. 2. Effects (mean Hedges' *d* effect sizes [ESs]) of herbivore chemical defenses on predators and parasitoids and variation related to enemy diet specialization. Positive ESs indicate effective defenses, while negative ESs indicate an increased susceptibility to enemies. Dots indicate mean ESs; horizontal lines denote 95% CI; sample sizes are shown in parentheses. The effect is significant if the 95% CI does not include zero. Significant (P < 0.05) Q_B values indicate between-group heterogeneity.

We obtained data on the effectiveness of chemical defenses for 53 species of Lepidoptera, 43 species of Coleoptera, 20 species of Hemiptera, 16 species of Hymenoptera, and five species of Orthoptera. These prey species or their defensive compounds were tested in different combinations against 11 parasitic species (9 insect and 2 nematode) and 113 predatory species: 35 vertebrates (23 birds, 7 mammals, 3 amphibians, and 2 reptiles) and 78 invertebrates (29 ants, 18 spiders, 8 bugs, 6 ladybirds, 4 wasps, 3 carabid beetles, 2 mantids, 2 syrphid flies, 2 lacewings, and 1 species each of bushcricket, antlion, centipede, and earwig). Research efforts were distributed unevenly among predatory groups: studies were dominated by birds (23.7% of ESs in our database) and ants (22.0% of ESs). Ten papers considered multispecies complexes of natural enemies.

Data on physiological costs of defenses were obtained for 10 species of Lepidoptera, seven species of Coleoptera, three species of Hymenoptera, and two species of Hemiptera. Studies evaluating costs of defenses preferentially explored herbivores sequestering defensive compounds (19 species, 32 publications); costs of *de novo* synthesis of chemical defenses were measured for three species only and reported in only one publication.

Effectiveness of defenses

Overall effect.—On average, chemical defenses of herbivorous insects were highly effective against natural enemies (d = 1.55, CI_{95} = 1.41 to 1.70, n = 354), and the effectiveness of defenses showed substantial heterogeneity across the studies (Q_T = 471.0, df = 353, P = 0.00003).

Variation related to characteristics of natural enemies.—In general, the herbivore chemical defenses were highly effective against predators and not effective against parasitoids (Fig. 2). Specialist predators were not affected by defensive secretion and specialist parasitoids even benefited from defensive secretions (Fig. 2), i.e., chemical defenses increased herbivore susceptibility to specialist parasitoids. In contrast to generalist predators, generalist parasitoids were not affected by chemical defenses of prey (Fig. 2).

The strength of defenses against different predatory species varied greatly (Q_B = 224.9, df = 48, P < 0.0001). The chemical defenses were more effective against vertebrate predators than against invertebrate predators (Fig. 3) and were most effective against birds (birds vs. other vertebrates: Q_B = 5.37, df = 1, P = 0.02). The effectiveness of prey chemical defenses also varied among orders of invertebrate predators; bugs showed, on average, no responses to prey chemical defenses (Fig. 3). Invertebrate predators using visual and chemical cues were similarly affected by prey defenses (Q_B = 0.62, df = 1, P = 0.43).

The effectiveness of defenses depended on the experience of both vertebrate and invertebrate predators, albeit in a different way (Fig. 4). In vertebrates, aversion of defended prey was higher in non-naïve predators (experienced or wild-caught) than in naïve predators (Fig. 4; Q_B = 9.94, df = 1, P = 0.0016). Responses of wild-caught vertebrate predators to the chemical defenses of a prey were similar to responses of predators that had learned to avoid this type of prey (Fig. 4; Q_B = 0.06, df = 1, P = 0.80). In contrast, the responses of wild-caught invertebrate predators were similar to the responses

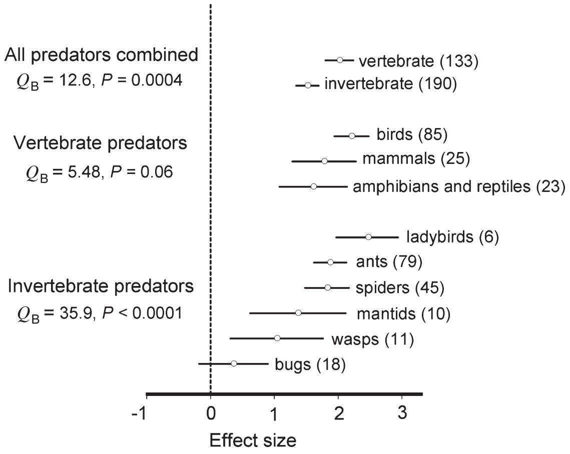

FIG. 3. Effects of herbivore chemical defenses on major taxa of predators (represented by at least four effect sizes). For explanations, consult Fig. 2.

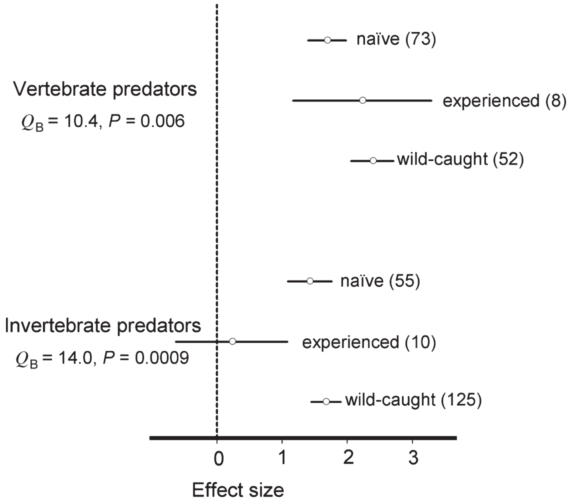

FIG. 4. Effect of predator experience on the effectiveness of herbivore chemical defenses (studies with predators having an unknown history were excluded from this analysis). For explanations, consult Fig. 2.

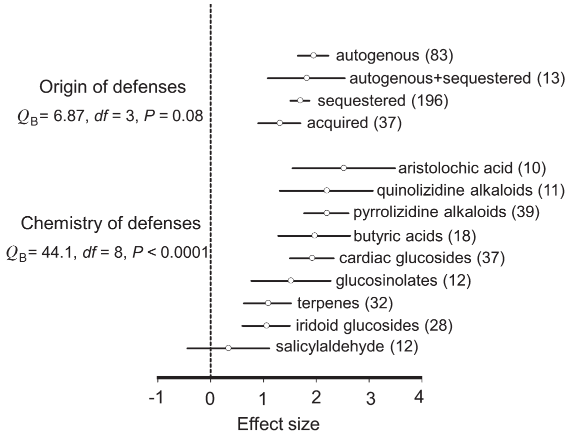

FIG. 5. Effects of origin and chemical composition of the defense compounds on their effectiveness against predators. (Only compounds presented by at least ten effect sizes were included.) For explanations, consult Fig. 2.

of naïve predators ($Q_{\mathrm{B}} = 1.56$, df = 1, $P = 0.21$), while prey defenses were not effective against predators that obtained experience in the earlier experiments with this prey (Fig. 4).

Variation related to prey characteristics.—Herbivores sequestering defensive chemicals from their host plants, synthesizing their defenses *de novo*, and possessing both defensive strategies were similarly defended against predators (Fig. 5; $Q_{\mathrm{B}} = 2.12$, df = 2, $P = 0.35$), while the acquisition of plant allelochemicals (i.e., their uptake without significant bioaccumulation) was a less effective strategy than sequestration (i.e., uptake with accumulation; Fig. 5; $Q_{\mathrm{B}} = 3.57$, df = 1, $P = 0.05$). Different defensive chemicals varied in their effectiveness against predators, with salicylaldehyde showing a nonsignificant effect (Fig. 5). Compounds with toxic properties and compounds with deterrent/repellent properties only were similarly effective against both vertebrate ($Q_{\mathrm{B}} = 1.11$, df = 1, $P = 0.29$) and invertebrate ($Q_{\mathrm{B}} = 1.10$, df = 1, $P = 0.29$) predators.

Externalized and non-externalized (internal) defenses, on average, were similarly effective (Fig. 6; $Q_{\mathrm{B}} = 0.05$, df = 1, $P = 0.82$); however, defensive secretions expelled by prey were more effective than internal defenses against naïve vertebrate predators ($Q_{\mathrm{B}} = 3.40$, df = 1, $P = 0.05$). At the same time, the expelling of defensive secretions led to higher parasitism rates compared with non-externalized defenses (Fig. 6). Types of externalization differed in their effectiveness (Fig. 6): expelling secretions and reflex bleeding in response to predator attack were similarly effective, whereas regurgitation and deposition of chemicals in fecal shields were less effective ($Q_{\mathrm{B}} = 13.04$, df = 1, $P = 0.0003$), most likely because 73.3% of species with the two latter strategies (but none of the species using the first two strategies; $\chi^2 = 62.0$, df = 1, $P < 0.0001$) acquired plant chemicals without their accumulation, thus producing less effective defenses than species that sequestered their defenses or synthesized them *de novo* (Fig. 5).

We found no differences among the five insect orders (Lepidoptera, Coleoptera, Hemiptera, Hymenoptera and Orthoptera) in the effects of their defenses on predators ($Q_{\mathrm{B}} = 5.24$, df = 4, $P = 0.26$). The defenses of all developmental stages were similarly effective against vertebrates ($Q_{\mathrm{B}} = 4.40$, df = 2, $P = 0.10$), but adult insects were better defended against invertebrate predators ($Q_{\mathrm{B}} = 12.5$,

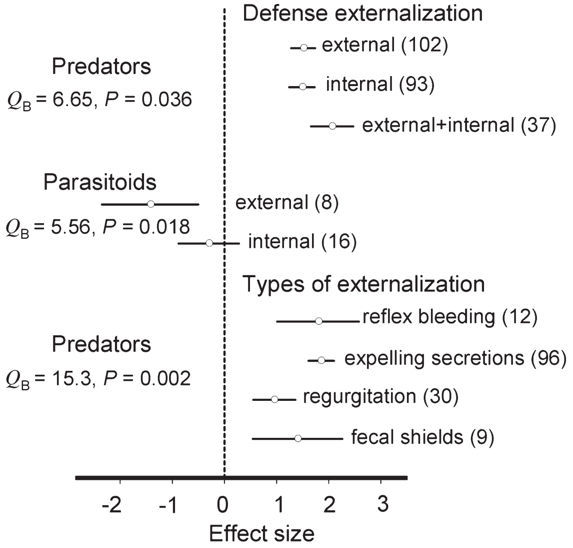

FIG. 6. Effects of externalization of chemical defenses on predators and parasitoids in tests with alive prey. For explanations, consult Fig. 2.

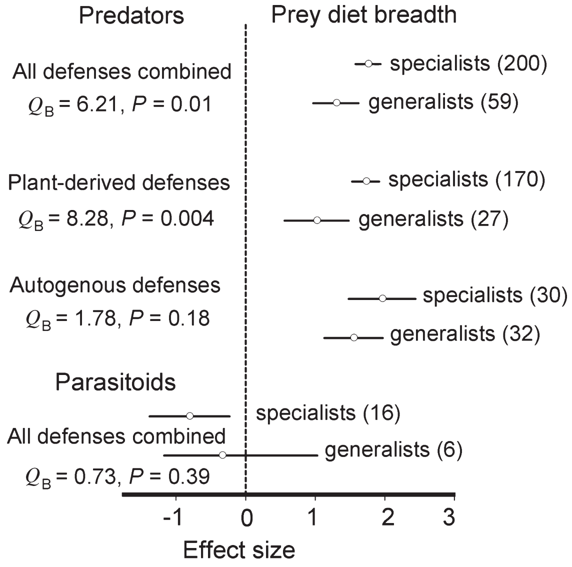

FIG. 7. Effects of chemical defenses of specialist and generalist herbivores on predators and parasitoids. For explanations, consult Fig. 2.

df = 1, P = 0.0004) than pre-imaginal stages, which did not differ in defense effectiveness (Q_B = 3.07, df = 2, P = 0.22).

Specialist herbivores more frequently derived their defenses from their host plants than generalist feeders (78.1% and 45.9%, respectively; χ^2 = 3.92, df = 1, P = 0.048). Defenses of specialist herbivores were more effective against predators than defenses of generalists, but this difference was mostly due to plant-derived defenses, while autogenous defenses showed similar efficacy in specialists and generalists (Fig. 7). At the same time, defenses of specialized herbivores increased their susceptibility to parasitoids, whereas defenses of generalists neither increased parasitism rates nor provided any protection from parasitoids (Fig. 7).

In three-quarters of the studied species, chemical defenses were accompanied by different types of warning displays, including acoustic signals and true aposematic and other conspicuous coloration. Warning displays were more frequently observed among species with internal defenses than among species with externalized defenses (64.8% and 35.9%, respectively; χ^2 = 3.92, df = 1, P = 0.048), but the proportion of true aposematic plus other conspicuous species was similar within specialist and generalist herbivores (75.2% and 67.6%, respectively; χ^2 = 0.82, df = 1, P = 0.36). Herbivores having true aposematic coloration and acoustic aposematism were equally defended against vertebrate predators (Q_B = 0.80, df = 1, P = 0.37), whereas effectiveness of defenses was higher for species with true aposematic coloration than for species with non-conspicuous or even other conspicuous coloration (Fig. 8A). The latter difference remained significant when only naïve predators were analyzed (Fig. 8B; Q_B = 6.47, df = 2, P = 0.04). The effectiveness of chemical defenses was lower against naïve than against non-naïve vertebrate predators within both true aposematic and other conspicuous prey species (Fig. 8B). In contrast to vertebrate predators, color aposematism did not affect effectiveness of chemical defenses against visually orienting invertebrate predators (Fig. 8A), both naïve and non-naïve (Fig. 8B).

Species with externalized defenses were more frequently gregarious than species with internal defenses (34.9% and 11.1%, respectively; χ^2 = 6.12, df = 1, P = 0.01). The effects of gregariousness were explored using the data from field experiments only because in the majority of laboratory experiments, gregarious species were tested singly. Field experiments demonstrated that gregarious species were better defended than solitary species (Q_B = 3.70, df = 1, P = 0.05). Morphological defenses, such as hairs and spines, and active defensive behavior,

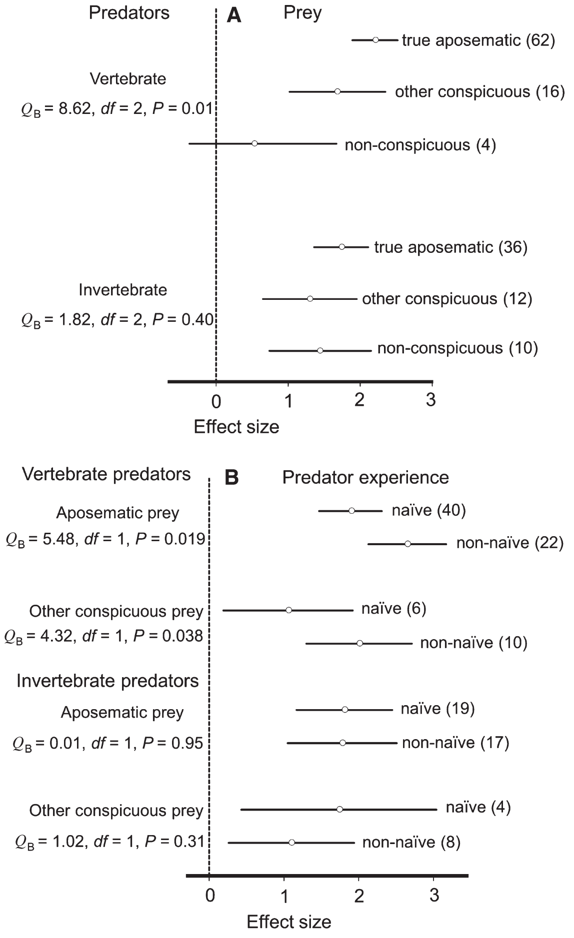

FIG. 8. Effects of prey warning coloration on the effectiveness of the prey's chemical defenses against vertebrate predators and visually hunting invertebrate predators in tests with alive prey: (A) all predators combined and (B) variation related to predators' experience (non-naïve: wild-caught and having experience with the same prey). For explanations, consult Fig. 2.

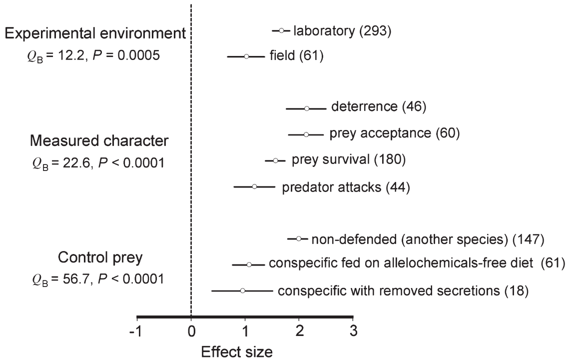

Fig. 9. Effects of experimental methods on the reported effectiveness of insect chemical defenses against predators. For explanations, consult Fig. 2.

such as thrashing and wriggling, did not enhance the effects of chemical defenses; in the experiments with live prey, survival was similar for prey having and lacking morphological defenses (Q_B = 0.81, df = 1, P = 0.37) or defensive behavior (Q_B = 0.23, df = 1, P = 0.63).

Variation related to methodology.—Laboratory experiments revealed much greater effects of herbivore chemical defenses compared with field experiments (Fig. 9). Field experiments with single species of predator yielded greater effects than experiments with multispecies complex of predators (Q_B = 5.07, df = 1, P = 0.02). Defenses of prey appeared considerably more effective when tested against another (non-defended) prey species than when tested against individuals of the same species lacking defenses (either fed on food lacking allelochemicals or with depleted secretion; Fig. 9). The highest values of effectiveness were obtained when researchers estimated the deterrence of defensive compounds and prey acceptance by predators (i.e., proportion of prey consumed), whereas studies that estimated prey survival yielded lower effectiveness, and the lowest values were obtained when predator attacks were recorded (Fig. 9).

Physiological costs of defenses

On average, chemical defenses of herbivorous insects did not incur any physiological costs for herbivores (d = 0.27, CI_{95} = 0.11 to 0.44, n = 87) and revealed substantial heterogeneity across the studies (Q_T = 138.6, df = 86, P = 0.0003). Sequestration generally improved herbivore performance, whereas three species that produced defensive secretions *de novo* demonstrated low, albeit significant, costs (Fig. 10). However, physiological costs of *de novo* synthesis of chemical defenses did not differ from costs of sequestration (Fig. 10), even when they were measured by same method (Q_B = 0.45, df = 1, P = 0.50). The costs of sequestration of different groups of plant allelochemicals were similarly nonsignificant (Fig. 10) and did not depend on the measured performance characteristic (weight, survival, developmental time, growth rate, fecundity; Q_B = 4.27, df = 4, P = 0.37). The costs of externalized and non-externalized defenses did not differ (Q_B = 0.14, df = 1, P = 0.71). The main source of variation was the method used to estimate the costs of defenses. Studies that measured costs as correlations of herbivore performance indices with the concentrations of sequestered chemicals in the insect body revealed no costs and even showed a positive association of performance with sequestration (Fig. 10) when herbivores were reared on both plants and artificial diets (Q_B = 0.52, df = 1, P = 0.47). Studies that intensified secretion production by the regular depletion of secretions did not reveal costs either but also did not demonstrate positive effects of secretion production on herbivore performance (Fig. 10). The difference between these two groups of studies remained significant when methods were compared within prey with externalized defenses (Q_B = 7.57, df = 1, P = 0.006). When herbivores producing salicylaldehyde (a special case of energy-gain defense production) were excluded, costs of other externalized defenses measured by depleting secretions became significant (d = −0.29, CI_{95} = −0.59 to −0.12, n = 9).

Discussion

Representativeness of the data used in the meta-analysis

A large number of primary studies on chemical defenses of insects did not fit our selection criteria. Still, our databases include all major groups of herbivorous prey and their natural enemies, major groups of defensive chemical compounds, and main strategies of

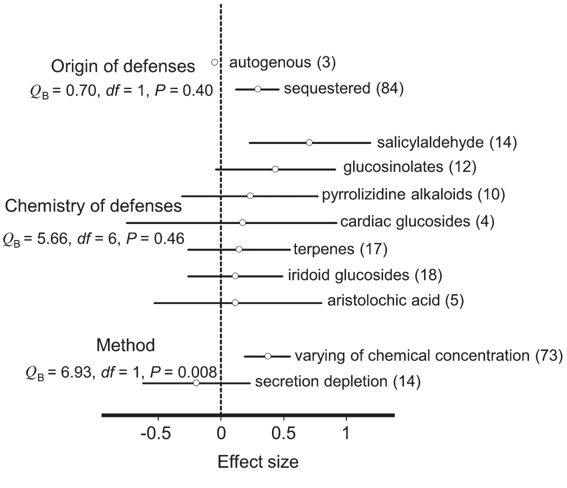

Fig. 10. Effects of defense origin, chemical composition of the defensive compounds (only compounds presented by at least four effect sizes were included), and experimental method on the reported physiological costs of defenses. For explanations, consult Fig. 2.

chemical defenses. The numbers of ESs in our databases (354 and 83 for effectiveness and costs, respectively) are considerably higher than in the majority of ecological meta-analyses (for examples, consult Côté and Reynolds 2012). The uneven distribution of data among the groups of predators, with a predominance of ants among invertebrates and of birds among vertebrates, presumably reflects the importance of these groups of predators in nature. Thus, our databases are sufficiently large and representative for explorations of diverse sources of variation in both the effectiveness and the costs of defenses.

We found that the reported magnitudes of adverse effects of defenses on natural enemies decreased substantially with the publication year. During the first period of intensive studies of chemical defenses, the researchers mostly collected supportive evidence for the effects of various compounds (with presumably defensive functions) on a limited number of predatory species (20 species were used in studies published from 1958 to 1986), primarily those that demonstrated clear responses to the defenses of prey. Later on, chemical defenses were tested against a larger number of natural enemies (111 species used from 1987 to 2014), and these studies revealed a high variability of responses: along with enemy species demonstrating strong aversion of defended prey, some enemy species were found to tolerate or overcome chemical defenses of a prey (Glendinning 2007) or even use defensive secretions to locate a prey (Köpf et al. 1997, Zvereva and Rank 2004). This may explain the discovered temporal trend in the reported magnitudes of adverse effects of defenses on natural enemies, which is in line with general decrease in the strength of any scientific finding with time; accumulation of disconfirming evidence commonly leads to reformulation of the original hypothesis and/or to restriction of its scope (Leimu and Koricheva 2004), and our meta-analysis contributes to this process.

Defense effectiveness against different enemies

We found that while chemical defenses protect herbivores from predators, they simultaneously increase prey mortality from parasitoids. This pattern, detected across several taxa of herbivores and their enemies, supports the generality of the observation made for 266 species of Neotropical moths and butterflies that species whose larvae contained defensive chemicals were preferentially parasitized (Gentry and Dyer 2002). The parasitoids may have evolved the ability to use chemically defended herbivores because these herbivores are less likely to be attacked by predators; thus, they provide enemy-free space for parasitoid larvae (Gentry and Dyer 2002). To locate the defended prey, specialized parasitoids have developed the ability to use defensive compounds as search cues (Mattiacci et al. 1993, Schaffner and Müller 2001, Zvereva and Rank 2004). Our meta-analysis suggests that this ability is a general phenomenon for specialist but not for generalist parasitoids, most likely because generalists use search cues other than the specific chemistry of a prey. Still, generalist parasitoids are able to overcome the chemical defenses of a host, as indicated by the absence of effects of host defenses on parasitism rates (Fig. 2). Thus, chemical defenses against predators have ecological costs expressed in increased infestation by specialist parasitoids, and the overall effects of chemical defenses against a complex of natural enemies depends upon whether predators or parasitoids are the main sources of mortality for a certain prey population. The local differences in the pressure from different enemies may create a mosaic of chemical defense strategies among geographic populations within one species (Gross et al. 2004*b*, Geiselhardt et al. 2015).

Predatory species differ in their responses to prey chemical defenses, and many of them can avoid, tolerate, or overcome these defenses (Krall et al. 1999, Glendinning 2007, Trigo 2011). We demonstrated that this variation is partly explained by the predator's dietary specialization: specialized predators, in general, showed no aversion to chemically defended prey (Fig. 2). Furthermore, some specialist predators, similarly to specialist parasitoids, use defensive secretions as a search cue (Köpf et al. 1997, Gross et al. 2004*b*). The finding that birds (which are predominantly generalist predators) are most strongly deterred by chemically defended prey could suggest that chemical defenses (frequently linked with warning displays) have evolved primarily as protection against birds. On the other hand, higher effectiveness of chemical defenses against vertebrate compared to invertebrate enemies may indicate that invertebrate enemies evolve in response to prey defenses faster than vertebrate predators; as a result, they have developed numerous adaptations to tolerate or overcome prey defenses better than vertebrates do.

Among invertebrate predators, only bugs (although they are mostly generalist predators) were not affected by prey defenses. Experiments showed that predatory bugs are not deterred by defensive chemicals in prey hemolymph (Boevé and Müller 2005) and can avoid contact with defensive chemicals in particular by puncturing the prey with their beaks away from defensive glands (Rank and Smiley 1994). Our result confirms the opinion (Boevé and Müller 2005) that bugs are the most severe predators of chemically defended invertebrates due to behavioral adaptations and efficient detoxification and/or excretion mechanisms.

To conclude, extremely high variation in the effectiveness of prey defenses against enemy species suggests that they exert different pressure on chemically defended herbivores. This diversity of predator responses may explain lower overall protective power of chemical defenses for some prey species in their natural environments against multispecies complexes of predators.

Learning and aposematism

Encounter by naïve predators with chemically defended prey may considerably enhance predator aversion. Aversion learning in vertebrates, which increases prey survival during subsequent encounters, is a widespread phenomenon confirmed in numerous studies with birds (Järvi et al. 1981, Staples et al. 2002, Svádová et al. 2009), lizards (Krall et al. 1997), and mammals (Whitman et al. 1986). Our finding that chemical defenses are more effective against experienced predators compared with naïve predators suggests that learning to avoid unprofitable prey is a general phenomenon in vertebrates. Aposematic signals play an important role in this process because they heighten wariness, accelerate learning, and enhance recognition and memorability (Ruxton et al. 2004). We found that the overall effectiveness of chemical defenses depends not only on the existence of a warning signal but also on its type: true aposematic colors and patterns (i.e., red, orange, and yellow, frequently forming contrasting patterns with black) enhance the effects of chemical defenses more than other types of conspicuous coloration (for example, monotonous black or metallic) against vertebrate predators. Thus, our analysis, which covered a wide range of color patterns, strategies of chemical defenses, and variety of predatory species, demonstrated that a true aposematic pattern is a much stronger signal accelerating learning and enhancing recognition and memorability in predators compared to other types of conspicuous coloration. This result is in line with experiments showing that internally contrasting patterns (Dolenská et al. 2009, Aronsson and Gamberale-Stille 2013), as well as red and orange colors (Svádová et al. 2009, Pegram and Rutowski 2014), are more effective than other patterns and colors in creating associations between the coloration and the unpalatability of prey. Interestingly, acoustic aposematism and true aposematic coloration are warning signals of similar strength, exceeding the strength of signal from other conspicuous coloration. This may indicate that acoustic and true color aposematism have evolved primarily in connection with chemical defenses against predators, while other types of conspicuousness are not necessarily linked with these defenses.

Our meta-analysis provided strong support for the generality of innate avoidance or unlearned wariness of aposematic prey by naïve vertebrate predators. The novel and interesting finding is that, similarly to experienced predators, naïve predators more strongly avoid prey with true aposematic coloration than prey with other conspicuous coloration. This finding is in line with studies showing that certain colors have a higher intrinsic aversive value than others (Jetz et al. 2001) and that aposematic patterns rather than conspicuousness cause innate avoidance (Lindström et al. 1999). This result also supports the hypothesis that the design of aposematic displays utilizes general properties of predators' cognitive systems (Ruxton et al. 2004) because avoidance of red and orange is likely genetically determined, and these colors may be a general signal of threat and intimidation in animals (Pryke 2009).

We found that predators experimentally educated to avoid a certain prey demonstrated an aversion similar to the aversiveness of this prey by wild-caught predators, although wild-caught predators were unlikely to have encountered the same prey species in nature. Thus, previous experience with various unprofitable and/or aposematic prey facilitates predator aversion against novel defended prey. This result suggests high abilities of predators for broad generalizations (Lindström et al. 2006) and supports the conclusions of some experiments with birds that imperfect Müllerian mimicry (low similarity between mimics) may be as effective against predators as perfect mimicry (Rowe et al. 2004, Lindström et al. 2006).

Generalization of experience with defended prey may also occur in the absence of aposematic signals, in particular because many defensive compounds share gustatory characteristics, such as bitterness, which serve as reliable signals of prey toxicity and cause avoidance in many vertebrates (Skelhorn and Rowe 2010, and references therein). On the other hand, high effectiveness of defenses against wild-caught predators may be explained not only by learning of certain colors and tastes but also by increased wariness of these predators due to previous encounters with multiple defended prey (Ruxton et al. 2004 and references therein).

In contrast to vertebrates, naïve and wild-caught invertebrate predators were similarly affected by prey defenses, presumably due to the low ability of invertebrates to learn and generalize information on prey defenses and/or coloration. Aversion learning has been demonstrated for spiders (McIver and Lattin 1990) and mantids (Paradise and Stamp 1991), but it seems to work only against a certain type of prey, and experience is not generalized. Moreover, many studies showed a higher acceptance of known prey, even a defended one, by some invertebrate predators, such as wasps or sawflies (Pasteels and Grégoire 1984, Rayor and Munson 2002). Our meta-analysis demonstrated the generality of the latter pattern: in contrast to vertebrates, experience in invertebrate predators leads to increased acceptance rather than to increased avoidance of defended prey.

Some visually hunting invertebrates (wasps, spiders, dragonflies, mantids) possess color vision and sometimes demonstrate learning of colors (VanderSal and Hebets 2007) and even avoidance of aposematic colors (Taylor et al. 2014). Still we found that warning coloration of prey in general does not increase the effectiveness of chemical defenses against invertebrate predators. Absence of the effects of warning coloration, even on visually hunting invertebrate predators, can be explained by low visual capabilities, by perception of visual signals from prey motion rather than from coloration, and by low abilities for associative learning (but see Taylor et al. 2014). We conclude that generally weaker effects of chemical defenses on invertebrate compared to vertebrate predators discovered in our meta-analysis most likely result from differences in the perception and learning of aposematic signals. Thus, our results do not support the hypothesis based on the case study by Taylor et al. (2014) that invertebrate enemies exert a strong influence on the evolution of warning signals in chemically defended prey.

Enemy responses to various defensive chemicals

Chemical composition of insect defenses is extremely variable, and this variability is thoroughly described in a number of reviews (Blum 1981, Pasteels et al. 1983, Opitz and Müller 2009). However, the effectiveness of defenses of different origin and of different chemical composition has been compared only rarely (but see Rowell-Rahier et al. 1995). Several studies demonstrated that defenses sequestered from herbivores' host plant are more effective than autogenously produced secretions (Rowell-Rahier et al. 1995, Zvereva et al. 2010*a*); however, in some cases this difference may have arisen from higher production of sequestered secretions relative to autogenous secretions (Gross et al. 2004*a*, Geiselhardt et al. 2015). Our meta-analysis showed no differences in the effectiveness of defenses of different origin across a variety of prey–predator systems. On the other hand, defenses derived by an herbivore from its food plant without accumulation appeared to impose lower overall effects than both sequestered (with accumulation of compounds) and autogenously produced defenses. We conclude that derivation of plant allelochemicals without their accumulation (observed mostly when chemicals are contained in regurgitant or deposited in fecal shields) is a less efficient strategy than sequestration accompanied by accumulation.

Different groups of chemicals used by herbivores for their defenses are effective against predators, although the magnitude of the effect varies. The only compound that demonstrated no significant effect was salicylaldehyde (Fig. 5), mostly due to adaptation of some specialist predators which, similarly to parasitoids, use this volatile compound as a cue for prey search (Köpf et al. 1997, Gross et al. 2004*b*, Zvereva et al. 2010*a*). Although this low effectiveness of salicylaldehyde may be compensated by energetic benefits associated with the release of glucose in the course of sequestration (Rowell-Rahier and Pasteels 1986), high pressure from specialist invertebrate enemies may explain evolutionary shifts from sequestering salicylaldehyde to autogenous production of butyrate-based defenses, as observed in some leaf beetles (Gross et al. 2004*b*).

We found no support for the hypothesis (Pasteels et al. 1983) that noxious defensive compounds are more effective against vertebrates, while volatile repellents act mostly against invertebrate predators: both groups of compounds were equally effective across experiments with both groups of predators. This result may be explained in several ways. First, many noxious compounds also act as deterrents affecting the chemical receptors of both vertebrates and invertebrates, thus providing a first line of defense against natural enemies. Therefore, attacked prey can be rejected by a predator and released unharmed (Wiklund and Järvi 1982, Sillén-Tullberg 1985) before toxic effects can occur. Many compounds used as chemical defenses share a bitter taste, which serves as a signal of toxicity (Skelhorn and Rowe 2010, and references therein) and prompts a predator to reject a prey based on experience with another type of defensive compound. Second, non-toxic volatiles may be highly irritating for vertebrates (Conner et al. 2007). Finally, the experiments included in our meta-analysis only considered the immediate effects of defensive chemicals on predator behavior, not the delayed toxic effects. Thus, although the toxic properties of defensive chemicals are known to favor aversion learning in vertebrate predators, the deterrence of these chemicals is more important for individual prey survival than their toxicity. We suggest that the toxic properties of the defensive compounds most likely have a relatively low importance for the evolution of chemical defenses because they do not affect the survival of individual prey. The existence of noxious defenses in a prey population may be supported only via kin selection, whereas compounds with deterrent properties may have evolved through both individual and kin selection.

Variation related to prey traits

Several case studies found that larvae of specialist Lepidoptera were better protected against predatory ants (Dyer 1995), paper wasps (Bernays 1988), and birds (Singer et al. 2014) compared with generalist species. However, the cited papers consider both chemically defended and non-defended prey species. Therefore, the detected differences were generally attributed to the more frequent use of plant allelochemicals by specialized herbivores for their own defenses (Bernays and Cornelius 1989, Dyer 1995). We found not only that specialist herbivores sequester defenses more frequently than generalists, but also that the defence effectiveness of specialist is higher than that of generalists. The latter result could not be explained by more frequent use of autogenous defenses by generalists compared to specialists (which mostly use sequestered defenses) because our meta-analysis demonstrated similar effects of sequestered and autogenous secretions (Fig. 5). Also aposematism, which increases the effectiveness of chemical defenses, was similarly frequent among generalist and specialist herbivores. We found, however, that difference in defense effectiveness between specialist and generalist herbivores is mostly due to species deriving defensive chemicals from their host plants. Therefore, we suggest that better defenses of specialists detected by our meta-analysis are at least partly explained by their ability to sequester larger amounts of plant allelochemicals compared with generalists, as previously demonstrated for specialist and generalist populations of the leaf beetle *Chrysomela lapponica* (Geiselhardt et al. 2015) and for specialist and generalist species of cyanogenic *Heliconius* butterflies (Engler-Chaouat and Gilbert 2007).

Our analysis of chemically defended species strongly supports the hypothesis that predators and parasitoids exert opposite selective pressures on the evolution of diet specialization in plant-feeding insects, which is frequently associated with sequestration of plant allelochemicals (Bernays and Graham 1988, Gentry and Dyer 2002). Predators select for narrowing the feeding niche of their herbivorous prey because diet specialization increases not only the likelihood of the development of chemical defenses in a prey but also the effectiveness of these defenses. In contrast, parasitoids support a broadening of herbivore host range because the dietary specialization of prey increases the probability of its encountering parasitoids that use defensive secretions as search cues. Importantly, we demonstrated for the first time that these contrasting impacts are associated not with the differences between predation and parasitism as trophic strategies but with the predominance of different levels of dietary specialization within these groups of enemies; the proportion of specialists among parasitoids is higher than among predators.

The externalization of defenses, i.e., the ability to release defensive chemicals upon predator attack, may be an important strategy in the evolution of chemical defenses; however, its role has so far received little attention (but see Higginson et al. 2011). The benefits of defense externalization are evident because prey can prevent an attack by repelling a predator from a distance or deterring a predator during an attack before being mortally damaged. Moreover, aversion learning of externalized defenses is more rapid compared with the aversion learning of defenses stored in the body (Skelhorn and Rowe 2006, Svádová et al. 2013). These benefits are confirmed by the existence of numerous strategies of externalization of chemical defenses, which have independently and repeatedly evolved in several insect taxa (Ohkuma et al. 2004). The most widespread strategy of externalization, expelling defensive secretions from specialized glands, is characteristic of many groups of insects, in particular bugs and leaf beetle larvae (Pasteels et al. 1983, Aldrich 1988). Larvae of the tobacco hornworm, *Manduca sexta*, exhale nicotine through the spiracles (Kumar et al. 2014), while tortoise beetles incorporate plant allelochemicals in fecal shields (Vencl and Morton 1998). Many lepidopteran and sawfly larvae, as well as orthopterans, regurgitate their gut contents (containing plant allelochemicals) in response to predator attack (Higginson et al. 2011, and references therein). Insects that store their defensive compounds in the body can also demonstrate externalization, such as reflex (easy) bleeding, when hemolymph containing defensive compounds is released in response to disturbance (Boevé et al. 2013), or the deposition of defensive compounds in the integument (Nishida 2002, Trigo 2011) or wing scales (Rossini et al. 2003) occurs. We did not find support for the hypothesis that externalized chemical defenses are more effective than those stored in the body tissues against either vertebrate or invertebrate predators, possibly because both strategies use warning displays indicating prey unsuitability to avoid predator attack. Externalized defenses are advertised to predators by chemical signals, while internal defenses are advertised by color or acoustic aposematism, which, as we found, accompanies chemical defenses stored in the body more frequently than it accompanies externalized defenses.

On the other hand, the frequency and benefits of defense externalization could be underestimated in our meta-analysis because we attributed to internal defenses all the cases when chemical defenses were analyzed in the whole insect body. However, some proportion of the chemicals found by these analyses could be deposited in the integument because in studies where the integument was analyzed separately, the defensive chemicals were commonly detected in it (Montllor et al. 1991, Fürstenberg-Hägg et al. 2014). This may indicate that defense externalization by deposition of defensive compounds in integument is more widespread than previously thought because it may increase prey survival upon predator attack due to taste rejection by the predator before the prey is mortally damaged.

We found that expelling secretions accumulated in specialized glands provided better protection against naïve vertebrate predators than defenses stored in the body tissues. This may indicate that for naïve predators, the immediate irritating effects of chemicals during attack are especially important because they have not yet learned to use aposematic signals, while experienced animals avoid direct contact with defensive chemicals, being deterred by the aposematic signal. This suggestion is supported by the absence of these differences in invertebrates, which (section "Learning and aposematism") generally have limited abilities for aversion learning.

Importantly, we found that defense externalization considerably increases the risk of parasitism. This effect is observed evidently because externalized (frequently volatile) defenses can be used more easily as a distant search cue, while chemicals stored in the body of the prey can be perceived by parasitoids only upon contact.

Effects of experimental methods on the outcomes of primary studies

Different aspects of methodology appeared to represent important sources of variation in the magnitude of the effects of chemical defenses on natural enemies. In line with earlier meta-analyses (Zvereva et al. 2010*b*, Zvereva and Kozlov 2012), laboratory experiments detected stronger effects than field experiments. The main reason behind the greater effects found in laboratory experiments is that researchers frequently use such measures of effectiveness as repellence or deterrence of the compound or acceptability (palatability) of a prey, while field experiments usually measure prey survival. Prey palatability (acceptability), i.e., the proportion of prey items consumed by the predator, cannot be directly translated to the mortality of prey because prey can be attacked, killed, and then abandoned. Prey survival, which has immediate implications for natural selection and the evolution of defenses, is the most adequate measure of defense effectiveness, while prey deterrence and/or acceptability generally overestimate the benefits of defenses for prey.

Laboratory experiments also eliminate a substantial part of the natural variability and complexity that is present in field environments. For example, ants, routinely used in bioassays with defensive compounds (Boevé 2010), overcome prey defenses in more natural conditions due to collective foraging and communication between nestmates, while individual ants are significantly deterred (Codella and Raffa 1996). Furthermore, field experiments frequently consider a multispecies complex of natural enemies, which further decreases the overall effectiveness of defenses due to the avoidance, tolerance, or overcoming of these defenses by some enemy species (section "*Defense effectiveness against different enemies*").

The magnitudes of defense effects also depend on the type of control used by a researcher: comparisons of the defended prey species with another, non-defended prey species generally yielded higher estimates of defense effectiveness than comparisons with conspecific prey lacking chemical defenses. In the latter case, prey, although depleted of defensive compounds, may still have some defenses, or prey may be unpalatable due to chemicals not considered by the experimenter. The use of non-defended prey as a control better reflects the natural situation, where predators, which are mostly generalists, usually have non-defended prey as an alternative.

We conclude that many methods that are widely used in studying chemical defenses of insects overestimate the benefits of defenses in terms of prey survival in nature. This overestimation should be taken into account when assessing the selective pressure from natural enemies in the modeling of evolutionary processes.

Physiological costs and evolution of chemical defenses

It is generally presumed that the production and storage of chemical defenses incurs energetic costs because it competes for resources with other organism functions (Bowers 1992, Ruxton et al. 2004). However, our meta-analysis did not reveal physiological costs of chemical defenses in general, although *de novo* synthesis of defenses was found to have significant, albeit low, physiological costs. Costs of *de novo* synthesis are generally presumed to be higher than costs of sequestration (Bowers 1992, Fürstenberg-Hägg et al. 2014); however, they were measured for herbivores in only one study (Rowell-Rahier and Pasteels 1986), and present a considerable research gap. Therefore, our result of lack of statistically significant difference between the costs of *de novo* synthesis and sequestration should be viewed with caution.

Costs of sequestered defenses are often measured by raising larvae on plants or artificial diets with varying concentrations of secondary compounds, but it is methodologically difficult to isolate plant allelochemicals used for sequestration as the only factor varying among different diets and to separate the costs of sequestration from other effects of plant allelochemicals (Bowers 1992). Specialist herbivores are adapted to allelochemicals in their host plants. Therefore, a diet lacking these allelochemicals may adversely affect their performance, thus complicating the detection of costs. This can explain the positive correlation between insect performance indices and concentrations of sequestered compounds in the insect's body and/or diet discovered by our meta-analysis. In contrast, the depletion of externalized secretion (i.e., simulating predatory attack) causes some decreases in insect performance. These costs become significant when we exclude experiments with larvae of several leaf beetle species producing salicylaldehyde as a major defensive compound. This type of defense represents a special case, because degradation of plant-derived salicin to salicylaldehyde is accompanied by the release of glucose, thus providing an insect with additional energy source for growth (Rowell-Rahier and Pasteels 1986). Significant costs of other types of defense, estimated by depletion of released secretions, may be considered as costs of defense externalization; they result from considerable losses of body fluid and defensive compounds (Bowers 1992, Higginson et al. 2011), which do not occur in the case of defenses stored inside the body (with the exception of species demonstrating reflex bleeding). Thus, externalized defenses incur additional energetic costs for replenishment of expended defensive secretions, which, as we found, are counterbalanced by higher effectiveness of externalized defenses against naïve vertebrate predators. Thus, cost–benefit ratio is in general higher for externalized defenses compared with defenses stored in the body, because these two strategies do not differ in their effectiveness. However, costs of externalization may appear in nature only when the encounters of prey with natural enemies occur frequently.

Although we did not detect physiological costs of chemical defense across the published studies, this result does not necessarily imply a lack of allocation costs, because some types of costs, for example the constitutive costs of possessing the specialized morphological structures and biochemical machinery of detoxification and sequestration of plant allelochemicals (Bowers 1992, Cogni et al. 2012), are difficult to estimate experimentally. We conclude that although the existence of physiological costs for production of chemical defenses is commonly presumed, these costs appear to be lacking or low in plant-feeding insects. This result has significant implications for our understanding of the evolution of ecological interactions, in particular because, as claimed by Cogni et al. (2012), an absence of costs challenges a basic assumption of ecological and evolutionary studies on a trade-off in organisms' investments in defenses and in other functions affecting its fitness. In plants, the expression of trade-offs between growth and antiherbivore defenses strongly depends on both ecological context and experimental methods

(Koricheva 2002), and the generality of these trade-offs is sometimes questioned (e.g., Messina et al. 2002). Moreover, in the field of plant antiherbivore defenses, it is becoming increasingly clear that ecological costs of defenses may exceed allocation costs (Karban 2011). Our meta-analysis confirms this statement for herbivore chemical defenses against natural enemies: along with an absence of physiological costs, we discovered high ecological costs in terms of increased susceptibility to parasitoids. Still, the favorable cost–benefit ratio, i.e., high effectiveness for small expenditures, may partly explain the prevalence of chemical anti-predator defenses in insects.

Conclusions

Large amounts of accumulated information make it possible to not only explore numerous sources of variation in the effectiveness and costs of chemical defenses of plant-feeding insects but also to test the generality of various hypotheses that have been suggested to explain different aspects of the evolution of chemical defenses. The meta-analysis confirmed several of these hypotheses and sometimes allowed broadening or restricting of their scope. For example, we quantitatively confirmed that herbivore chemical defenses act in opposite direction on two major groups of natural enemies, predators and parasitoids, and concluded that chemical defenses, which have likely evolved under selection pressure from predators, bear high ecological costs in terms of increased parasitism. We found that chemical defenses of herbivorous insects are most effective against birds compared with not only invertebrate predators but also with other vertebrates. We found a strong influence of prey and enemy dietary specialization on the magnitude and, even, the direction of the defense effects. We confirmed for natural systems that prey species displaying aposematic signals are better defended against both naïve and experienced vertebrate predators, and we found that true aposematic coloration is more effective for both aversion learning and innate avoidance than other types of conspicuousness. Externalization enhanced the protective value of chemical defenses only against naïve vertebrates. In contrast, we found no support for the hypothesis that toxic compounds are effective mostly against vertebrate enemies, while repellent volatiles are effective mostly against invertebrate enemies. An overall absence of physiological costs for the production of defensive chemicals questions the evolutionary role of trade-offs between costs and effectiveness of defenses.

Our meta-analysis revealed several research gaps: in particular, costs of defenses are studied less frequently than effects of defenses on natural enemies, and effects of defenses on parasitoids were explored in only a few studies, although parasitoids play an important role in herbivore population dynamics and evolution. The discovery of generally positive effects of defenses on parasitoids calls for more studies of the interactions between chemically defended herbivores and their parasitoids, simultaneously incorporating the effects of predators on both herbivores and parasitoids. While the physiological costs of defense sequestration have received some attention, the costs of *de novo* synthesis remain nearly unexplored. Our meta-analysis revealed considerable effects of research methods on the reported values of physiological costs and suggested that the exploration of costs of defenses requires further development of methodology. More data on physiological and ecological costs of anti-predator defenses are needed to further our knowledge of the evolution of chemical defenses in insects.

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