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Influence of Pollen Nutrition on Honey Bee Health: Do Pollen Quality and Diversity Matter?

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Abstract

Honey bee colonies are highly dependent upon the availability of floral resources from which they get the nutrients (notably pollen) necessary to their development and survival. However, foraging areas are currently affected by the intensification of agriculture and landscape alteration. Bees are therefore confronted to disparities in time and space of floral resource abundance, type and diversity, which might provide inadequate nutrition and endanger colonies. The beneficial influence of pollen availability on bee health is well-established but whether quality and diversity of pollen diets can modify bee health remains largely unknown. We therefore tested the influence of pollen diet quality (different monofloral pollens) and diversity (polyfloral pollen diet) on the physiology of young nurse bees, which have a distinct nutritional physiology (e.g. hypopharyngeal gland development and *vitellogenin* level), and on the tolerance to the microsporidian parasite *Nosema ceranae* by measuring bee survival and the activity of different enzymes potentially involved in bee health and defense response (glutathione-S-transferase (detoxification), phenoloxidase (immunity) and alkaline phosphatase (metabolism)). We found that both nurse bee physiology and the tolerance to the parasite were affected by pollen quality. Pollen diet diversity had no effect on the nurse bee physiology and the survival of healthy bees. However, when parasitized, bees fed with the polyfloral blend lived longer than bees fed with monofloral pollens, excepted for the protein-richest monofloral pollen. Furthermore, the survival was positively correlated to alkaline phosphatase activity in healthy bees and to phenoloxydase activities in infected bees. Our results support the idea that both the quality and diversity (in a specific context) of pollen can shape bee physiology and might help to better understand the influence of agriculture and land-use intensification on bee nutrition and health.

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Introduction

By ensuring reproduction of many plants, pollinators, like honey bees, are essential to the functioning of natural and agricultural ecosystems [1–3]. In turn, pollinators benefit from this pollination service by harvesting the nutrients (nectar and pollen) required for their growth and health. For example, in honey bees, floral nectar, containing carbohydrates and stored as honey, is the energetic fuel of individuals, and pollen provides most of the nutrients required for their physiological development [4]. The development and the survival of honey bee colonies are therefore intimately associated with the availability of those environmental nutrients [4–6], which suggests that the alteration of bee foraging areas due to the current intensification of agriculture and landscape changes might provide a deficient nutrition and therefore affect honey bee populations [7,8]. This is further supported by beekeepers, who are ranking poor nutrition and starvation as two of the main reasons for colony losses [9]. Therefore, studying the link between nutrient availability and bee health might help to better understand the current bee losses observed throughout the world [10,11].

Among those flower nutrients, pollen, which is virtually the main source of proteins, amino acids, lipids, starch, sterols, vitamins and minerals [12,13], is a major factor influencing the longevity of individuals [6]. Pollen is also important at the colony level, since it enables the production of jelly by young workers, that is used to feed larvae, the queen, drones and

older workers [14,15]. Therefore, a direct consequence of nutritional deficiency (pollen shortage) is a decrease in the colony population [5] and likely a deficient health of individuals, which could also affect the resistance threshold of bees to other stress (pathogens or pesticides) [8,16]. Indeed, pollen intake is known for influencing the physiological metabolism [17,18], immunity [19], the tolerance to pathogens like bacteria [20], virus [21] and microsporidia [22] and reducing the sensitivity to pesticides [23]. However, honey bees rarely face a total lack of pollen in their environment but are rather confronted with variability in time and space of pollen resource abundance, type and diversity. In addition, pollens can differ between floral species regarding their nutritional contents [24–26] suggesting that some are of better quality for bees than others. Therefore, studying the influence of pollen intake on bee health requires also taking into account the quality and diversity of pollen diets. Despite some studies showed that pollen quality can affect the longevity of bees [27–30] and the hypopharyngeal gland development [29,31] and, more recently, that pollen diversity might improve some immune functions [19], our knowledge of the influence of quality and diversity of pollen diets on bee health is rather limited.

To improve our knowledge on this topic, the influence of pollen diet quality and diversity was tested on nurse physiology and the tolerance to a parasite. Since pollen is essentially consumed by young nurse bees, they have a very specific nutritional physiology with large lipid and protein stores (see 32,33 for reviews). Notably, pollen intake enables the development of their hypopharyngeal glands, where digested pollen nutrients are used to produce jelly, a proteinaceous glandular secretion shared with nestmates [14,15]. Nurse bee physiology was thus assessed by determining the development of the hypopharyngeal glands but also the gene expression level of *vitellogenin*, which is highly expressed in nurses as compared to foragers [34], and encodes a major protein produced in the fat body and used for jelly production [35]. This gene, that can be nutritionally regulated [17,18], also slows down aging [36] and is involved in the regulation of cellular immune functions [37]. We included the analysis of the gene *transferrin*, an iron transport protein also produced in the fat body, and involved in ovary development [38–40] and immune response [41], like *vitellogenin*. However, it is not known whether it is nutritionally regulated, which will be tested through this study. Finally, the tolerance to parasitism was tested using the highly prevalent microsporidia Nosema *ceranae*, a gut parasite that might play a role in colony losses or honey bee weakening [42–45]. For that purpose, we assessed the effects of pollen diet and parasite on bee survival and on physiology by measuring the activity of glutathione-S-transferase (GST), phenoloxidase (PO) and alkaline phosphatase (ALP). GSTs are important in the detoxification of endogenous and exogenous compounds [46] and can be induced in insect gut after bacterial infection, suggesting a protective role against pathogens [47]. In addition, previous studies showed a higher GST activity after *Nosema* infection in bees [48,49]. PO plays an important role in insect immunity by encapsulating pathogens (e.g. bacteria and fungi) and repairing tissues via melanogenesis [50], and ALP, involved in many metabolic processes, is highly expressed in insect gut and plays a pivotal role in intestine health in mammals [51].

Materials and Methods

Pollen diet composition and nutritional factors

The effects of pollen quality and diversity were tested by feeding bees with monofloral diets that differed regarding their nutritional properties or a polyfloral diet composed of the different monofloral pollens. Four blends of wild flower pollens with a respective predominance of *Cistus, Erica, Castanea* and *Rubus* pollens were purchased fresh from Pollenergie® (France) and stored at -20° C. Pollen pellets were collected from pollen traps at the hive entrance. Monofloral pollen diets of *Cistus, Erica, Castanea* and *Rubus* were obtained by sorting by color the pellets of the predominant pollen from each blend. Palynological tests were then performed to validate the genus of each sorted pollen. The polyfloral pollen diet was composed of a mixture of the four monofloral pollens (25% of each according to their weight).

To assess the nutritional quality of each pollen diet, we analyzed their protein, amino acid, lipid and sugar contents, as well as their antioxidant capacities. The protein content was determined by microkjeldahl analysis (N x 6.25) using a Vapodest 45 (Gerhardt) and according to the procedure ISO 5983-2 [52]. Total lipids were analyzed after the disruption of pollen wall using an acid hydrolysis with hydrochloric acid (HCl 6N). Then lipids were extracted with a chloroform/methanol mixture (2:1, v/v) following the method of Folch et al. [53]. The protein and lipid contents were expressed as percent of dry matter, which was determined after drying the pollen for 24 h at 75° C [54]. The nature and the concentrations of amino acids were determined in 20 mg of pollen with the ion-exchange chromatography technique using an automated amino acid analyzer according to the procedure EC 152/2009 [55]. The Oxygen radical absorbance capacity (ORAC) method with AAPH (2,2'-azobis(2-amidino-propane) dihydrochloride) as a free-radical generator was used, as described by Ou et al. [56], to measure the antioxidant capacity in 1 g of each pollen. The antioxidant trolox was used as a standard and thus the data expressed as trolox equivalent. To qualitatively measure sugar contents the pollens were dehydrated for 48 h at 35° C. Thirty mg of pollen were weighed and 1000 µl of Ultrahigh-quality water (18.2 mΩ) were added. The content was passed with a Hamilton syringe through a 0.2 µm filter (Millex LG CI, 0.2 microns; Millipore) and injected into HPAEC Dionex ICS- 3000 equipment. Separation of carbohydrates was carried out on a CarboPac PA-1 guard column (4 x 50 mm) and a CarboPac PA-1 anion-exchange column (4 x 250 mm) after two-fold dilution. The quantitative determination of carbohydrates was carried out by pulsed amperometric detection [57]. The presence of pesticide residues in the different pollen diets was assessed via gas and liquid chromatography with a limit of quantification of 0.01 mg/kg and a limit of detection of 0,005 mg/kg following the AFNOR 15662 procedure [58] (List of analyzed pesticides in Table S1).

Bee rearing and feeding

To control the pollen intake, the experiments were performed on 1-day-old bees (*Apis mellifera*) reared in cages (10.5 cm x 7.5 cm x 11.5 cm). Age-matched bees were obtained by placing honeycombs containing late-stage pupae into an incubator at 34° C and 50-70% of humidity, and collecting bees that emerged within 10 hours. They originated from three colonies and were mixed before placing them in cages. The caged bees, kept in an incubator (34° C and 50-70% of humidity), were provided *ad libitum* with candy (Apifonda + powdered sugar) and water. Groups of bees were fed with one of the following monofloral pollen diets: *Erica, Cistus, Rubus* or *Castanea*, a mixture of the four pollens (polyfloral diet) or did not receive any pollen. Pollen diets were prepared by mixing pollen with water at the mass ratio of 10/1 (pollen/water) and were freshly prepared and replaced every day for 7 days. To prevent a potential nutritive compensation of bees fed with one of the pollen diet, they were not provided with *ad libitum* pollen but with determined quantity of pollen each day: 4 mg/bee the first two days, 5 mg/bee the next two days, 3 mg/bee the fifth day, and 2 mg/bee the last two days. Those quantities were determined through preliminary experiments and represent the minimal consumption of all pollens on each day; and as previously found pollen consumption varies with age of the bees (increased the first days and then decreased) [4,31]. Using this method, bees were provided with the same quantity of each pollen diet and consumed all of it on each day. Since some bees died during the pollen feeding period (7 days), the pollen quantities were adjusted each day to the number of surviving bees.

Influence of pollen quality and diversity on bee nurse physiology

Groups of 35 one-day old bees were placed in cages and reared for 7 days with one of the pollen diet. On day 8, they were flash frozen in liquid nitrogen and stored at -80° C for subsequent physiological analyses. The experiment was repeated 14 times per pollen diet.

Development of hypopharyngeal glands. The right and left glands form of five bees per cage were dissected on ice in 100 µl of physiological serum (0.9% NaCl). Both glands were slide-mounted and analyzed under an optical microscope coupled to a CF 11 DSP camera (Kappa). The gland development was assessed by measuring the maximum diameter of 15 randomly chosen acini per gland (n = 30 acini per bees) [59] with the Saisam 5.0.1 software (Microvision®).

Abdomen gene expression. The abdomens of 10 bees per cage were homogenized in 1 ml of Trizol reagent (Invitrogen®) with a TissueLyser (Qiagen®) (4 x 30 s at 30 Hz). The mixture was incubated for 5 min at room temperature and after centrifugation (12,000 g for 30 s at 4° C) 500 µl of the supernatant was used for RNA extraction. One hundred µl of Chloroform (Sigma®) were added and the solution was incubated for 3 min and centrifuged (12,000 g for 15 min at 4° C). The aqueous phase was mixed with an equal volume of 70% ethanol (Sigma®) and transferred into a Qiagen RNeasy column. RNA extraction was carried out as indicated in the Qiagen RNeasy kit for total RNA with on-column DNase I treatment (Qiagen®). For cDNA synthesis, 1,000 ng of RNA per sample were reverse-transcribed using the High capacity RNA to cDNA Kit (Applied Biosystems). cDNA samples were diluted ten-fold in molecular grade water.

The expression level of *vitellogenin* and *transferrin* was determined by quantitative PCR using a StepOne-Plus Real-Time PCR Systems (Applied Biosystems®) and the SYBR green detection method including the ROX passive reference dye. Three µl cDNA were mixed to 5 µl SYBR Green PCR Master Mix (Applied Biosystems®), 1 µl of forward primer (10 µmol) and 1 µl of reverse primer (10 µmol) of candidate genes. Cycle threshold (Ct) values of selected genes were normalized to the housekeeping gene *Actin* using the comparative quantification method (delta Ct method). Primer sequences (5'–3') were: *vitellogenin* forward: TTGACCAAGACAAGCGGAACT, reverse: AAGGTTCGAATTAACGATGAA [60]; *transferrin* gene: forward: AGCGGCATACTCCAGGGAC, reverse: CGTTGAGCCTGATCCATACGA [61]; *Actin* forward: TGCCAACACTGTCCTTTCTG, reverse: AGAATTGACCCACCAATCCA.

Influence of pollen quality and diversity on bee tolerance to *Nosema ceranae*

For the experiment on bee tolerance to *Nosema ceranae*, groups of 70 one-day old bees were placed in cages and reared for 7 days with one of the pollen diet. For each pollen diet, one group was infected with *Nosema* and one group was *Nosema*-free, giving 12 treatment groups. On day 10, 28 bees per cage were flash frozen in liquid nitrogen and stored at -20° C until analysis of glutathione-S-transferase, alkaline phosphatase and phenoloxidase. The other 42 bees were used to determine the influence of pollen diet and *Nosema ceranae* on bee survival. Dead bees were counted daily and removed from the cages until half of the bees had died. The experiment was repeated 9 times per treatment group (pollen diet, *Nosema* infection).

Bee infection with *Nosema ceranae*. *Nosema* spores were isolated from infected colonies. Ten abdomens of forager bees were crushed in 2 µl of distilled water using an electric grinder (Ultra Turrax ® T18 basic, IKA®). Homogenates were then filtered with paper Whatman No. 4, and the filtrate was supplemented with 10 ml of distilled water. Solutions were centrifuged three times at 800 g for 6 minutes and each time the spore pellet was resuspended in 10 ml of distilled water. Species identification was performed as in Alaux et al. [62] and the spore concentration was determined using a haemocytometer. To equally infect bees with a *Nosema ceranae inoculum*, bees were fed individually with 2 µl of freshly prepared 50% sucrose solution containing 100,000 spores, which is known to cause an infection in worker bees [63–65]. Control bees were fed with a sucrose solution. At the end of the experiment, the guts of the bees were analyzed: no spores were found in the control bees but the infected bees were heavily parasitized (data not shown).

Enzyme analysis. Enzyme activities were assayed in different bee tissues: GST in the gut and head, ALP in the gut and PO in the abdomen devoid of gut. All analyses were

Table 1. Nutritional factor contents in the different pollen

Pollen diets	Proteins (%)	Lipids (%)	Sugars (%)	Amino acids (g)	Antioxidants (µmol)
Cistus	12	6.9	5.2	11.9	103
Erica	14.8	7.4	4.8	16.27	196
Castanea	21.6	6.6	5.0	18.68	399
Rubus	22	6.4	6.7	19.98	475
Mix	17.6	6.8	5.4	16.71	293

Mix indicates the pollen diet composed of 25% of each monofloral pollen. Pollen proteins, lipids and sugars are expressed as percent of pollen dry matter. The antioxydant power is expressed in µmol of Trolox equivalent/g of pollen. The amino acids are expressed in g/100g of pollen.

performed on 3 pools of 3 bees per cage and in triplicate. Samples were homogenized at 4° C with TissueLyser (Qiagen®) (5 x10 s at 30 Hz) in the extraction buffer (10 mM NaCl, 1% (w/v) Triton X-100, 40 mM sodium phosphate pH 7.4, containing a mixture of 2 mg/ml of antipain, leupeptin and pepstatin A, 25 units/ml of aprotinin and 0.1 mg/ml of trypsin inhibitor) based on the weight of each pool (10% w/v extract). The homogenate was then centrifuged at 4° C for 20 min at 15,000 g. The enzymatic activities in supernatant were assayed in microplates with a BioTek Synergy HT100 spectrophotometer (BioTek Instruments®). GST was assayed in a reaction medium (200 µL final volume) containing 10 µl of tissue extract and 1 mM EDTA, 2.5 mM reduced glutathione, 1 mM 1-chloro-2,4-dinitrobenzene and 100 mM Na/K-phosphate pH 7.4. GST activity was followed spectrophotometrically at 340 nm by measuring the conjugation of 1-chloro-2,4-dinitrobenzene with reduced glutathione for 5 min at 25° C. ALP was assayed in a reaction medium (200 µL final volume) containing 10 µl of tissue extract and 20 mM of $MgCl_2$, 2 mM of *p*-nitrophenyl phosphate as a substrate and 100 mM Tris-HCl pH 8.5 [66]. ALP activity was followed by measuring *p*-nitrophenyl phosphate hydrolysis at 410 nm for 5 min at 25° C. PO was assayed in a reaction medium (200 µL final volume) containing 50 µl of tissue extract and 200 mM NaCl, 0,4 mg/mL L-Dopa (3,4-Dihydroxy-L-phenylalanine), 100 mM sodium phosphate pH 7.2). PO activity was followed at 490 nm by measuring the conversion of L-Dopa to melanin for 10 min [62].

Statistical analysis. The statistical analysis was performed using the statistical software R [67]. Since the data were not normally distributed, the influence of pollen quality and diversity on hypopharyngeal gland development, *vitellogenin* and *transferrin* expressions, and enzymatic activities was analyzed using Kruskal-Wallis and Dunn's multiple comparison tests. To analyze survival data obtained during the 50 days of experiment, we transformed the data in survival table and the remaining bees were considered alive at the day 50. Consequently, we used a Cox proportional hazards regression model, with R functions (coxph) and the package [survival] [68] to analyze the effect of *Nosema*, pollen and Nosema x pollen interaction on bee survival. Then, the effects of *Nosema* for each pollen diet and the effect of each pollen in non- and *Nosema*-parasitized bees on survival were tested. For non- and *Nosema*-parasitized bees, the influence of pollen diets on enzyme activities was determined using Kruskal-Wallis and Dunn's multiple comparison tests. For each pollen diet, the effect of *Nosema* parasitism on enzymes activities was analyzed using Mann-Whitney U tests. Finally, in order to better understand the underlying mechanisms of bee longevity, we assessed the link between LT50 (day on which 50% of the bees had died in each cage based on the raw data) and enzyme activities (average value of the 3 analyzed pools per cage) using Spearman correlation for healthy and parasitized bees.

Results

Pollen diet nutritional factors

The nutritional value of each pollen was characterized before testing their effects on bees (Table 1). We did not detect the presence of pesticides in the four pollens that composed the different diets (Table S1). Contrary to lipids and sugars, the levels of proteins, amino acids and antioxidant capacity varied greatly between pollens. Therefore, pollen diets could be ranked according to their protein content as follows (from the poorest to the richest): *Cistus*, *Erica*, Mix (25% of each pollen), *Castanea* and *Rubus*. Exactly the same trend was found when looking at amino acids and antioxidants levels. The difference between *Cistus* and *Rubus* was especially striking with the latter having about twice as many proteins and amino acids, and almost five times greater antioxidant capacity. However, the lipid and sugar contents, which did not vary as much, followed different patterns. For example, *Erica* pollen was the richest in lipids but the poorest in sugars, and the other way round for *Rubus* pollen.

All pollen diets contained the same amino acids including the 10 essential amino acids required for the bee adult development [69]: arginine, histidine, lysine, tryptophan, phenylalanine, methionine, threonine, leucine, isoleucine, and valine (Table S2). As for protein contents, most amino acids were in lower amounts in the *Cistus* pollen (notably the 10 essential amino acids) and in higher amounts in the *Rubus* pollen, whereas *Erica* and *Castanea* pollens had intermediary levels. Only proline was at the highest amount in *Cistus* pollen.

Regarding individual sugars, only glucose and fructose were found in all pollens (Table S3). Trehalose, a major hemolymph sugar of bees, was present in *Cistus* and *Castanea* pollens. Finally, erlose was only found in Castanea pollen, which contained all analyzed sugars.

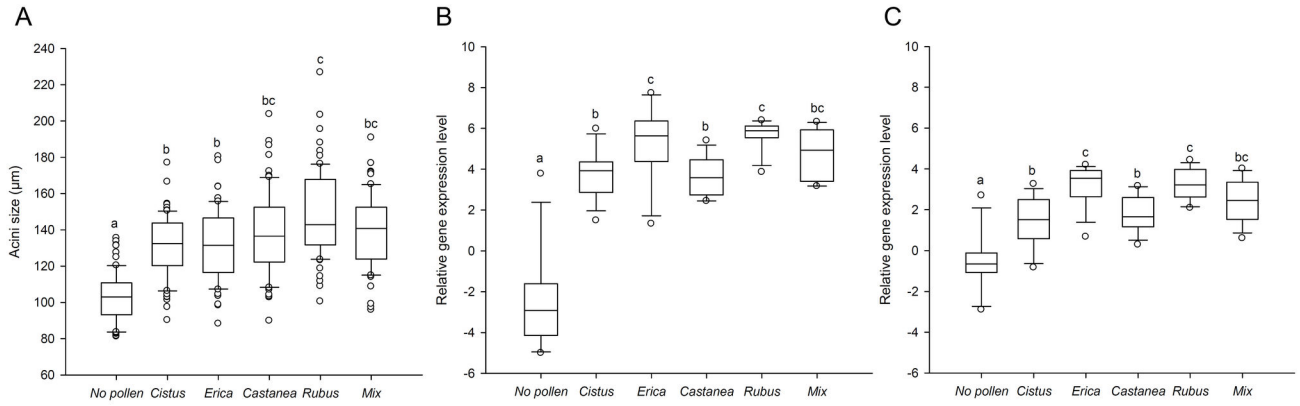

Figure 1. Effects of pollen quality and diversity on nurse physiology. (A) Size of hypopharyngeal gland acini, (B) *vitellogenin* and (C) *tansferrin* expression levels. Box plots are shown for 5 and 10 bees/replicate for the glands and each gene, respectively (n = 14 replicates giving 70 and 140 bees/pollen diet for the glands and each gene, respectively). Different letters indicate significant differences between pollen diets ($p < 0.05$, Kruskal-Wallis and Dunn's multiple comparison tests). Boxes show 1st and 3rd interquartile range with line denoting median. Whiskers encompass 90% of the individuals, beyond which each outliers are represented by circles.

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Influence of pollen quality and diversity on nurse bee physiology

Pollen feeding modified the hypopharyngeal glands development (Kruskal-Wallis test, H = 143.84, $p < 0.001$; Figure 1A), which was reduced in bees reared without pollen but varied depending on pollen quality, since acini were more developed in bees fed with *Rubus* pollen compared to bees fed with *Cistus* and *Erica* pollen (Figure 1A). The gland development of bees fed with the polyfloral blend was not different from bees provided with the monofloral diets (139.5 ± 2.3 µm) but was almost equal to the average gland development induced by the four diets (137.5 ± 4.1 µm).

The expression level of *vitellogenin* and *transferrin* was significantly affected by the different pollen diets (*vitellogenin*: Kruskal-Wallis test, H = 43.13, $p < 0.001$, Figure 1B; *transferrin*: Kruskal-Wallis test, H = 42.31, $p < 0.001$, Figure 1C), with a higher expression in bees fed with pollen than in bees that did not receive pollen (Figure 1C). Interestingly, the quality of pollen diet also shaped the expression of both genes since *Erica* and *Rubus* pollen triggered the highest expression of *vitellogenin* and *transferrin* (Figure 1B and C). The influence of the polyfloral diet was not different from that of the others diets (*vitellogenin*: 4.8 ± 0.3 and *transferrin*: 2.4 ± 0.3), and corresponded to the average gene expression level induced by the four monofloral diets (*vitellogenin*: 4.6 ± 0.5 and *transferrin*: 2.4 ± 0.5).

Influence of pollen quality and diversity on bee tolerance to *Nosema ceranae*

Nosema parasitism and pollen diets decreased and increased the survival of bees, respectively (Cox model, $p <$ 0.001 for each factor, Figure 2). *Nosema* effect was observed regardless of the type of pollen diet ($p < 0.001$ for each pollen diet, Figure 2) and pollen diets modified the survival of bees regardless of the exposure to *Nosema* (Figure 2 and Table 2). However, we found a significant interaction between *Nosema* and pollen diets ($p < 0.001$, Figure 2). Except for the *Cistus* pollen, the quality and diversity of pollen diet did not influence the survival of healthy bees, but it mattered when bees were parasitized (Figure 2 and Table 2). Indeed, we observed a significant hierarchical influence of monofloral pollens on the survival of parasitized bees with the following order from the least to the most beneficial pollen: *Cistus* < *Castanea* < *Erica* < *Rubus*. In addition, bees fed with the polyfloral pollen blend lived significantly longer than bees provided with *Cistus*, *Erica* and *Castanea* pollen but there was no significant difference with bees provided with *Rubus* pollen (Figure 2 and Table 2).

When looking at the bee physiology, *Nosema* did not affect gut GST activity (Figure 3A). However, pollen diets did modify GST level in both healthy and parasitized bees (Kruskal-Wallis test, H = 35.73, $p < 0.001$ and Kruskal-Wallis test, H = 32.73, $p <$ 0.001, respectively, Figure 3A) and the highest activity was observed with *Erica* pollen diet (Figure 3A). In the head, GST activity was significantly lower in bees infected with *Nosema* (Figure 3B) but was higher in bees fed with pollen regardless of exposure to *Nosema* (Kruskal-Wallis test, H = 22.06, $p < 0.001$ and Kruskal-Wallis test, H = 27.28, $p < 0.001$, respectively, Figure 3B). Contrary to what was observed in the gut, the type of pollen diet did not affect head GST level.

Nosema ceranae caused a decrease in ALP activity whatever the pollen diet (Figure 4). However, besides a higher activity induced by *Castanea* pollen compared to *Cistus* pollen in healthy bees, the quality and the diversity of pollen supply did not affect the ALP activity in the bee gut (healthy bees: Kruskal-Wallis test, H = 14.29, p = 0.013 and parasitized bees: Kruskal-Wallis test, H = 12.54, p = 0.028, Figure 4).

Nosema ceranae induced a significant increase of PO activity in bees deprived of pollen (Figure 5). In infected bees the immune enzyme activity was lower in the presence of

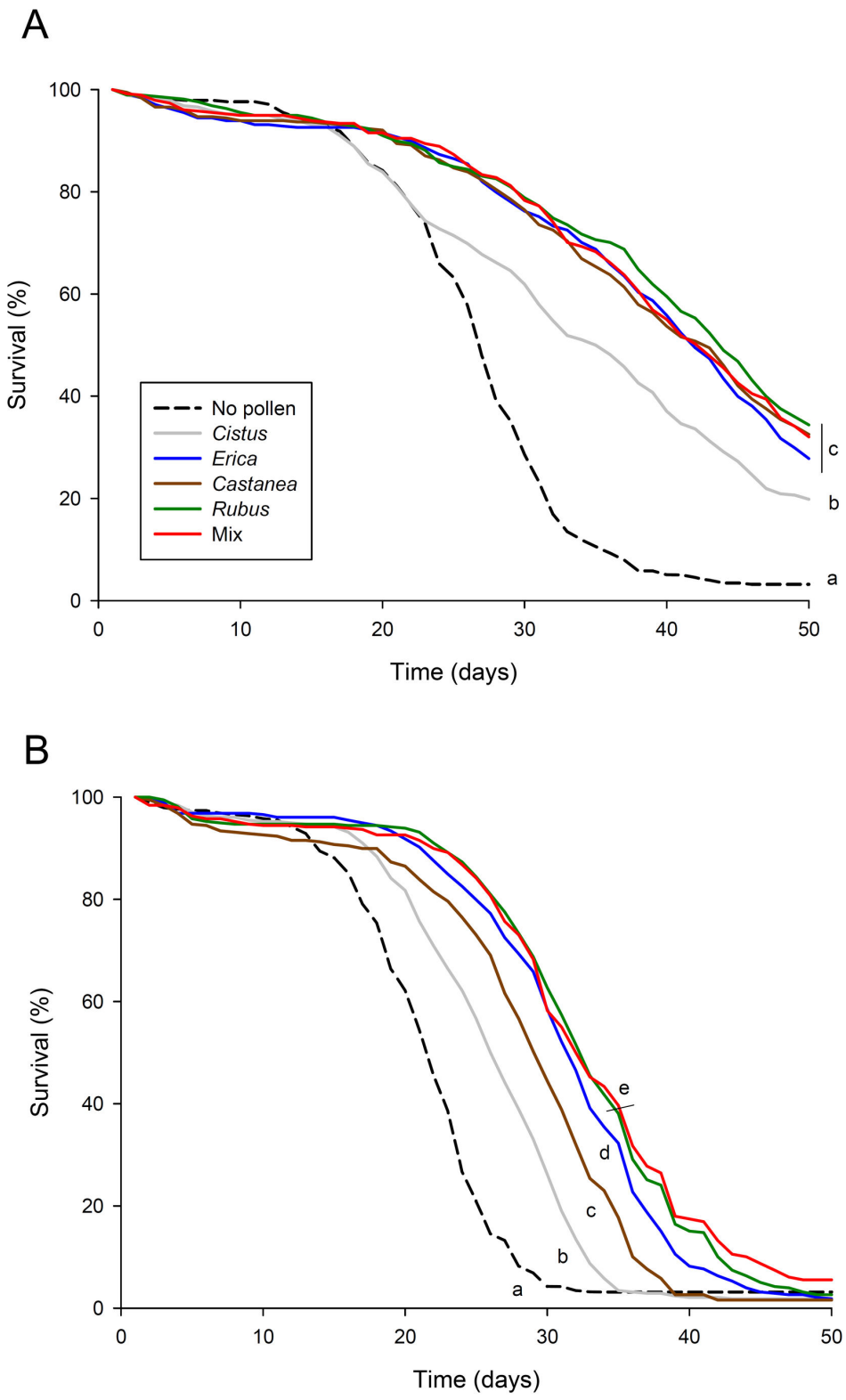

Figure 2. Effects of pollen diet and *Nosema ceranae* infection on bee survival. Data show the percentage of survival over 50 days for (A) non-parasitized and (B) *Nosema*-parasitized bees (9 replicates/pollen diet). Different letters denote significant differences between pollen diets in non-parasitized or *Nosema*-parasitized bees ($p < 0.05$, Cox proportional hazards regression model).

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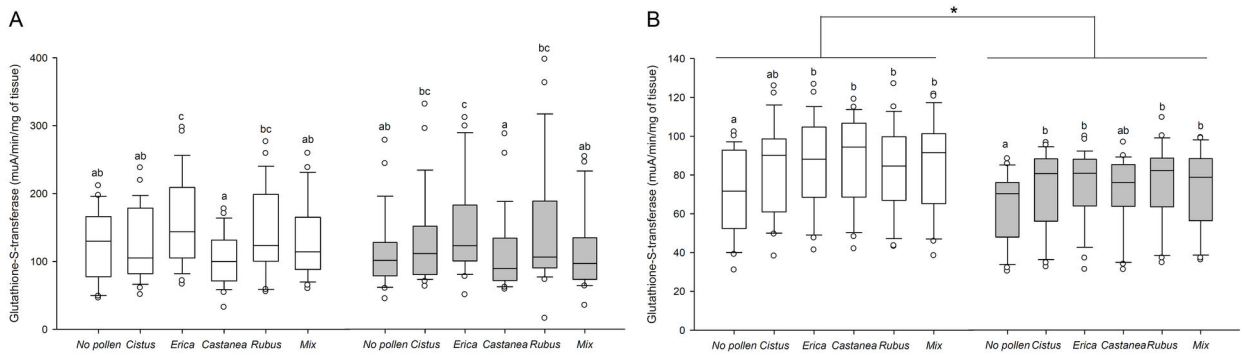

Figure 3. Effects of pollen diet and *Nosema ceranae* infection on glutathione S-transferase. The enzyme activity was assessed in (A) the guts and (B) the heads of bees. Box plots are shown for 3 pools of 3 bees/replicate (n = 9 replicates giving 81 bees total/pollen diet). Different letters denote significant differences between pollen diets in non-parasitized (white box plots) or *Nosema*-parasitized bees (grey box plots) ($p < 0.05$, Kruskal-Wallis and Dunn's multiple comparison tests) and * indicate significant differences between parasitized and non-parasitized bees for each pollen diet ($p < 0.05$, Mann-Whitney U tests). Boxes show 1st and 3rd interquartile range with line denoting median. Whiskers encompass 90% of the individuals, beyond which each outliers are represented by circles.

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Table 2. Comparative effects of pollen diets on the survival of (A) non- and (B) *Nosema*-parasitized bees.

	Cistus	*Erica*	*Castanea*	*Rubus*	Mix
A					
No pollen	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Cistus		< 0.0001	< 0.0001	< 0.0001	< 0.0001
Erica			0.47	0.1	0.35
Castanea				0.36	0.84
Rubus					0.47
B					
No pollen	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Cistus		< 0.0001	< 0.0001	< 0.0001	< 0.0001
Erica			< 0.0001	0.047	0.007
Castanea				< 0.0001	< 0.0001
Rubus					0.42

P-values from the Cox proportional hazards regression model are reported.

pollen, except for *Erica* (Kruskal-Wallis test, H = 49.64, $p < 0.001$, Figure 5). In healthy bees, pollen intake had limited effect on PO activity (Kruskal-Wallis test, H = 19.24, $p < 0.001$, Figure 5). Only *Erica* pollen elicited a significant higher activity when compared to *Castanea* and *Rubus* pollen.

Lastly, we determined whether the LT50 of bees was linked to the activity of the different investigated enzymes. In healthy bees longevity was positively correlated with ALP activity (i.e., ALP activity explained 50% of bee longevity), but when bees were *Nosema*-infected, longevity was positively linked to PO activity (Figure 6).

Discussion

The results of this study support the idea that the nutritional quality and diversity of pollen nutrition can shape bee health. Indeed, we found that both bee physiology and tolerance to a parasite varied depending on the type of pollen diet, suggesting that not only does the availability but also the quality of environmental resources matter.

The type of pollen provided to the bees had significant effects on the nurse bee physiology. Bees fed with the protein-richest pollen (*Rubus*) presented the most developed acini and the highest expression level of *vitellogenin* and *transferrin*. This tends to confirm previous studies that showed that the hypopharyngeal gland development is linked to the level of proteins in the diet [29,31]. However, the other pollen diets did not significantly induce different gland developments, which could be explained by a too small range of protein and/or other nutritional factors contents. Pollen feeding also increased the expressions of *vitellogenin* and *transferrin*. Since both genes are expressed in the fat bodies, the main site of nutrient storage, and pollen promotes the development of fat bodies [19], it is reasonable to expect an increase in both gene expression levels, as previously found for *vitellogenin* after consumption of proteins [70]. However, the expressions of *vitellogenin* and *transferrin* in bees fed with *Erica* pollen were not different from bees fed with *Rubus* pollen, although *Erica* had a lower amount of proteins. This suggests that their expression is not only sensitive to the protein level but also to other nutritional factors. When looking at the nutritional factors, we found that *Erica* pollen had the highest content in lipids, which might have promoted the increase of fat bodies and therefore the expression of both genes, since fat body tissues are also the primary site of lipid metabolism (e.g. fatty acid synthesis and triacylglyceride production) [71]. This potential

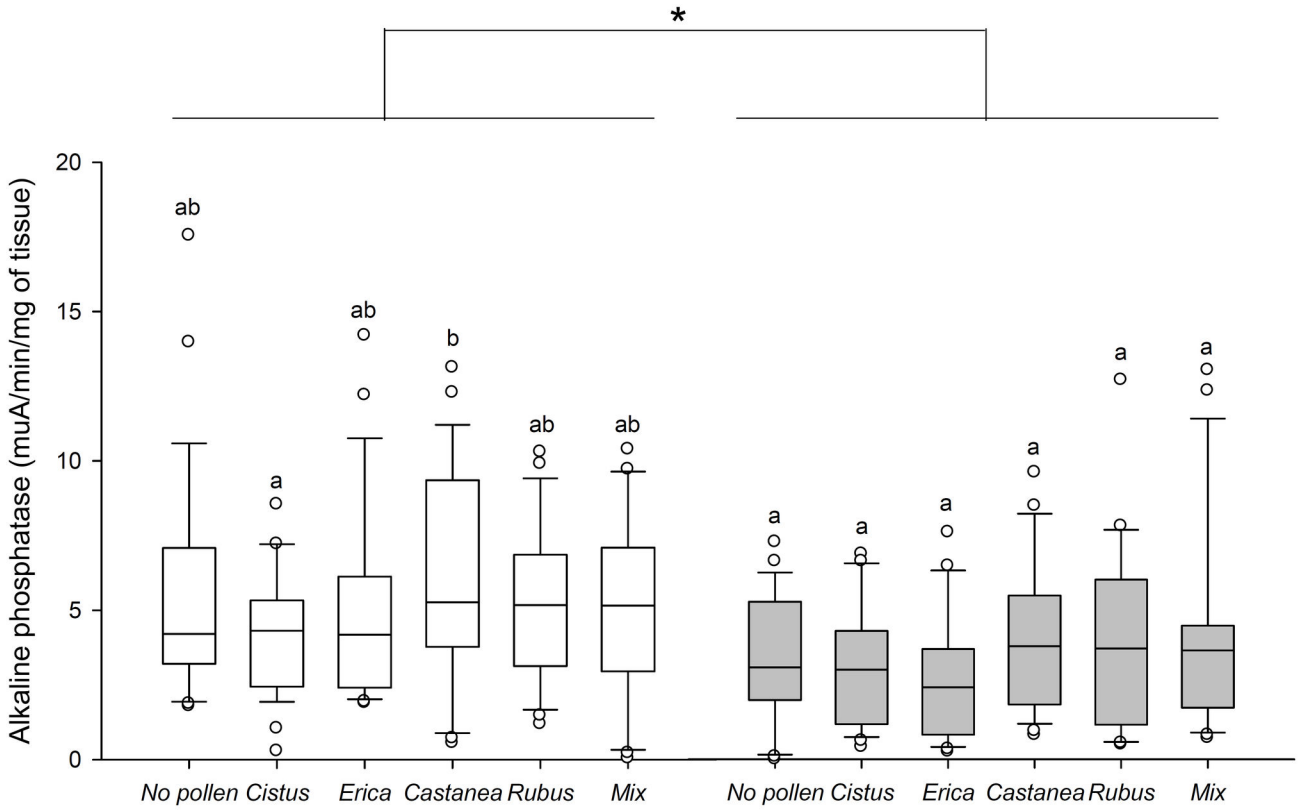

Figure 4. Effect of pollen diet and *Nosema ceranae* infection on gut alkaline phosphatase. Box plots are shown for 3 pools of 3 bees/replicate (n = 9 replicates giving 81 bees total/pollen diet). Different letters denote significant differences between pollen diets in non-parasitized (white box plots) or *Nosema*-parasitized bees (grey box plots) ($p < 0.05$, Kruskal-Wallis and Dunn's multiple comparison tests) and * indicate significant differences between parasitized and non-parasitized bees for each pollen diet ($p < 0.05$, Mann-Whitney U tests). Boxes show 1st and 3rd interquartile range with line denoting median. Whiskers encompass 90% of the individuals, beyond which each outliers are represented by circles.

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role of lipids in *vitellogenin* synthesis would further confirm that they are essential to the nurse physiology [72] and the production of brood [73]. In addition, it is interesting to note that *vitellogenin* and *transferrin* had similar expression patterns according to the different pollen diets. This covariation in gene expression was also found in previous works studying the potential role of those genes in ovary development [38,39].

The quality of pollen also influenced the tolerance of bees to a parasite (*Nosema ceranae*). As expected, infection by *Nosema* decreased the survival of bees [49,64] and pollen nutrition increased the survival of both healthy and parasitized bees. Except for bees fed with the protein-poorest pollen (*Cistus*), we did not observe a difference in survival between the different pollen diets when bees where non-parasitized. However, pollen quality had a strong influence when bees were parasitized by the microsporidia; the survival of bees was significantly different between the four different monofloral diets (from the least to the most beneficial pollen: *Cistus* < *Castanea* < *Erica* < *Rubus*). This suggests that the quality of pollen nutrients might have no or limited consequences on the physiology of bees when they are healthy, but it might affect their capacity to tolerate an external stress like parasites. The positive influence of *Rubus* pollen as compared to *Cistus* pollen has also been proved when looking at the effect of diet quality on larvae weight in bumble bees [74]. The extremely high protein and antioxidant levels of *Rubus* pollen, as compared to *Cistus* pollen, could explain the greater survival of infected bees fed with the former pollen. Notably proteins are known to improve bee survival (see 4 for a review). High levels of amino acids could play an important role too, since ten of them are essential to the bees in specific concentrations [69]. However, the hierarchical influence of monofloral diet was not linked to the protein, amino acid or antioxidant levels, e.g. bees fed with *Erica* pollen (14.8% of protein) lived longer than bees fed with *Castanea* (21.6% of protein). *Erica* pollen had actually the highest lipid content and promoted a higher production of *vitellogenin* than *Castanea* pollen (Figure 1B). The positive influence of *vitellogenin* on bee lifespan [36] might then contribute to the increased survival of parasitized bees supplied with *Erica* pollen. This suggests that the quality of pollen should not be estimated based on a single or few

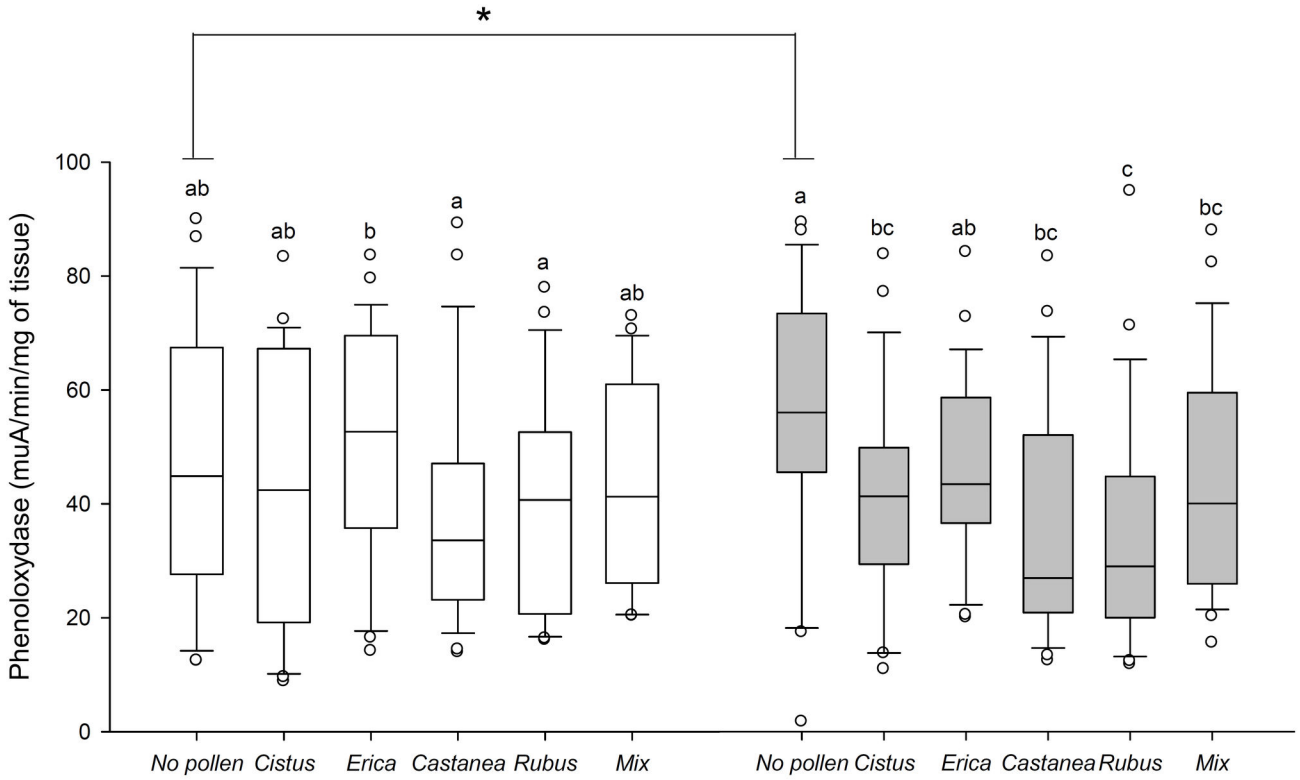

Figure 5. Effect of pollen diet and *Nosema ceranae* infection on phenoloxidase. Box plots are shown for 3 pools of 3 bees/replicate (n = 9 replicates giving 81 bees total/pollen diet). Different letters denote significant differences between pollen diets in non-parasitized (white box plots) or *Nosema*-parasitized bees (grey box plots) ($p < 0.05$, Kruskal-Wallis and Dunn's multiple comparison tests) and * indicate significant differences between parasitized and non-parasitized bees for each pollen diet ($p < 0.05$, Mann-Whitney U tests). Boxes show 1st and 3rd interquartile range with line denoting median. Whiskers encompass 90% of the individuals, beyond which each outliers are represented by circles.

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nutritional factors, but by taking all the nutritional factors as a whole.

Regarding the defense mechanism, the general activity of GST (detoxification), ALP and PO (immunity) changed too according to the pollen diets, but we did not observe a pattern similar to the bee survival. Therefore it was not possible to link the influence of diet quality on bee survival to the activity level of those enzymes. Moreover, the patterns of enzymes activity were not modified by *Nosema* infection, but the general level of head GST and ALP was reduced, confirming a previous study [49]. However, an increase of GST activity in the gut of *Nosema*-parasitized bees has been previously reported [48,49], likely to protect the host from the oxidative stress induced by the parasite [47]. The lack of GST response in our study could be due to the diet, since we did not use a commercial mixture of proteins, amino acids and vitamins as in both previous studies, which could have promoted a GST response. Interestingly, the activity profile of GST in the gut was very similar to the expression profile of *vitellogenin* and *transferrin* according to the different diets, with *Erica* and *Rubus* pollen giving the highest activity. However, nothing is known about the relationship between GST and those two genes. Regarding PO activity, it is well-known in other insects that PO level can be influenced by the diet quality [75–77]. Indeed, melanogenesis, regulated by PO through the synthesis of melanin (a nitrogen-rich quinone polymer), might be costly in nitrogen [78] and thus sensitive to variations in nitrogen resources. However, it did not vary between pollen diets in a previous study [19] and, in our study, it was only higher with *Erica* pollen. Therefore, further investigations are needed to better understand the relationship between pollen diet and PO activity in bees.

Pollen dietary diversity was not associated with an improvement of nurse physiology, as reflected by the measured physiological parameters. The influence of the polyfloral diet actually came down to the average of each monofloral pollen influence. This suggests that a high-quality monofloral pollen may be better than a mixture of lower nutritional quality as found for brood rearing [79,80]. However, it is likely that different physiological factors in bees are not affected equally by the pollen diet. This has been observed in a recent study showing a higher activity of glucose oxidase in bees fed with a polyfloral pollen blend as compared to monofloral pollen, but PO activity and hemocyte count were not

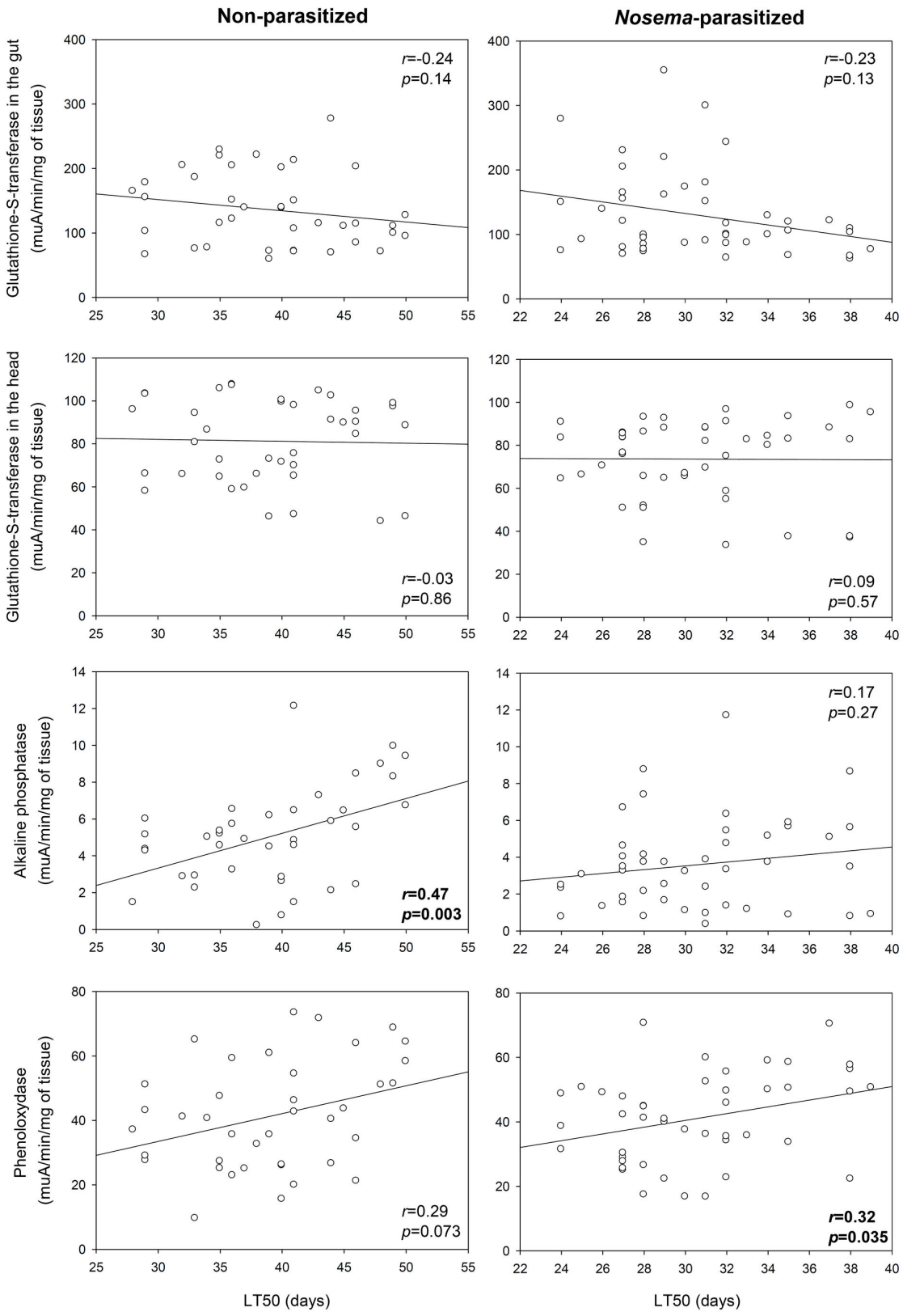

Figure 6. Correlations between LT50 and enzyme activities in non- and *Nosema*-parasitized bees. *r* and *p*-values are shown. LT50: day on which 50% of the bees had died in each cage.

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affected by the polyfloral diet [19]. This is further confirmed by our study, since the polyfloral blend had a positive influence on the survival of parasitized bees. It did not correspond to the average of each pollen effect, but was higher than *Cistus, Castanea* and *Erica* pollens and to the same level than *Rubus* pollen. This trend was not observed in healthy bees suggesting again that nutritional quality can significantly affect the susceptibility of individuals to parasites. It is not known whether the increase in the survival of bees fed with the polyfloral blend was due to the combination of the four pollens or the simple presence of *Rubus* pollen, although it contained a quarter of this pollen. Similar results were found by Foley et al. [81], who observed a decreased susceptibility to the fungal parasite *Aspergillus* of bee larvae fed with a specific pollen or with a mixture.

Finally, in order to decipher some of the underlying physiological mechanisms involved in bee health, we determined whether the activity of GST, PO and ALP were associated to an increase of survival in healthy or parasitized bees. Survival was positively associated to ALP and PO activity in healthy and *Nosema*-infected bees, respectively. In mammals, ALP is involved in the regulation of nutrient absorption (notably lipids), detoxification of bacterial lipopolysaccharide, intestinal tolerance to commensal bacteria, prevents bacterial invasion and reduces intestinal inflammation, playing thus a pivotal role in intestine health (see 51 for a review). It is not known whether ALP has similar roles in insects but there are structural and functional homologies between insect and mammal ALPs [82]. In addition, the correlation between ALP activity and bee survival suggests that this enzyme might be important in insect health. When its activity was decreased by *Nosema* infection, it was no longer linked to bee survival. In that case, the survival rate was associated to PO activity. However, except in the absence of pollen, parasitized bees did not mount a PO immune response, which supports the idea that the bee survival was simply linked to a higher basal activity of PO.

In conclusion, pollens are not equal regarding their effects on bee health and a polyfloral blend is not necessarily better than a monofloral pollen of good nutritional values (e.g. *Rubus* pollen). However, when bees are infected (by *N. ceranae* here), the availability of different floral resources can cover the limited influence of some pollens and improve the tolerance to the infection to the level of a rich pollen. Pollinating areas of bees are currently changing due to intensification of agriculture and landscape alteration, and bees are often confronted with decreasing availability and diversity of resources in time and space. Global climate change is also expected to modify the environmental resources of bees due to changes in plant phenology and distribution [83]. Therefore, maintaining and/or developing floral resources within agro-ecosystems is needed to prevent the negative impact of human activity and sustain the bee population [7].

Supporting Information

Table S1. List of pesticides analyzed in the pollen diets.
(XLSX)

Table S2. Amino acids present in the different pollens. Their concentration is expressed in g/100 g of pollen.
(DOCX)

Table S3. Sugars present in the different pollens. Their quantity is expressed in mg per g of pollen. *nd*: not detected and *nq*: present but not quantifiable.
(DOCX)

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Author Contributions

Conceived and designed the experiments: GDP YLC LPB AD CA. Performed the experiments: GDP MS SS CA. Analyzed the data: GDP AK JLB CA. Contributed reagents/materials/analysis tools: YLC LPB AD. Wrote the manuscript: GDP CA.

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