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Combining Geometric Morphometrics and population genetics to evaluate effects of persistent organic pollutants in Alpine Bumblebees

submitted by
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« In the end we will conserve only
what we love;
we will love only
what we understand;
and we will understand only
what we are taught. »

~ Baba Dioum, 1968 ~

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Data are freely available from the corresponding author (gurtensabrina@gmail.com).

Abstract

Insects face a major threat: In recent years, studies around the globe have reported huge declines not only in diversity but also in terms of biomass. Among the main drivers for this substantial insect loss are man-made, globally distributed pollutants. Some of these harmful substances, so called persistent organic pollutants (POPs), are traceable over decades — even far away from their original source — and therefore are of great concern. Atmospherically carried POPs get primarily deposited through precipitation. Mountainous regions are characterized by particularly high precipitation rates, resulting in a high deposition of POPs transported over long distances. Nevertheless, only a few field studies have yet addressed the toxicological effects of POPs on the health state and developmental integrity of alpine organisms. Here, the local accumulation of atmospherically measured POPs and mercury was assessed in two bumblebee species occurring above the tree line at Zugspitze (Germany) and at Hoher Sonnblick (Austria): *Bombus wurflenii* represents a typical species of high-altitude habitats, while *B. pratorum* displays a ubiquitous distribution across all altitudinal levels. We detected nearly half of all 77 persistent pollutants tested, as well as mercury, in all bumblebee samples. By means of population genetics we were able to disentangle genetic factors, like inbreeding, and environmental stressors affecting the two bumblebee species, as both stressors are potential fitness constraints. Coupled with the results from geometric morphometrics, we could confirm environmentally induced phenotypic changes in bumblebee wings: We found, with few exceptions, highly positive correlations for POPs or mercury and fluctuating asymmetry in the wing shape of *B. wurflenii*. In contrast, *B. pratorum* was less responsive to the pollution, presumably due to different strategies in feeding and nesting behavior. These findings emphasize the importance of species-specific chemical analyses to relate pollution levels to fitness proxies. In the context of the rapidly progressing global change, there is an urgent need to find a way to better protect and conserve alpine biodiversity across national borders.

***Bombus* spp. / pollution / POPs / microsatellites / fluctuating asymmetry**

1 Introduction

About 1.9 million animal species are currently described, whereof over a million belong to insects alone (Chapman, 2009). Insects thereby represent the largest and most diverse group within the animal kingdom (Grimaldi & Engel, 2005). Novel approaches estimate that another 4.5 to 6 million insect species still await discovery (Stork, 2018). As Robert M. May accurately summarized in 1986: „Indeed, to a good approximation, all species are insects." Even though the cumulative biomass of insects is comparatively low (Bar-On et al., 2018), they play a crucial role for the functioning of terrestrial and freshwater ecosystems (Weisser & Siemann, 2004; Yang & Gratton, 2014). By interlinking primary producers with higher trophic levels, insects are decisively important for the maintenance of whole food webs (Yang & Gratton, 2014). Beside the cohesion of whole ecosystem communities, insects ensure irreplaceable ecosystem services, like degradation of organic matter, biocontrol of pests, disease-vector control, and pollination of flowering plants (Rosenberg et al., 1986). Around 80% of the world's wild plants (Ollerton et al., 2011) and 75% of cultivated food crops (Klein et al., 2007) depend on insect pollination.

In the last decades, insects experienced a dramatical decline in biomass and abundance (Kluser & Peduzzi, 2007; Dirzo et al., 2014; Wagner et al., 2021). The rate of extinction is nearly eight times faster than that of mammals and birds (Sánchez-Bayo & Wyckhuys, 2019). A long-term monitoring in protected areas showed that more than 75% of the biomass of flying insects completely vanished (Hallmann et al., 2017). The diversity of insects is also strongly decreasing. A recently published review by Sánchez-Bayo & Wyckhuys (2019) expected about 40% of the world's insect species to be at risk for extinction in the next few decades. This global phenomenon affects rare species to a similar extent as generalists (Warren et al., 2020). The disappearance of such key ecosystem members leads to shifts and collapses of whole communities. The big threat to the most species-rich group of animals endangers the functioning of all ecosystems.

The main drivers of the worldwide insect decline are thought to be habitat loss through monoculture farming and urbanization, as well as pollution, mainly by the use of synthetic pesticides and fertilizers (Sánchez-Bayo & Wyckhuys, 2019). In addition, biological factors, like invasive species or pathogens and climate change destructively impact insect populations. In many habitats, these stressors act simultaneously. Certain combinations thereof are highly detrimental and lead to an untenable burden for exposed individuals, particularly susceptible species, or even whole communities.

One group of pollutants reached exceptional scientific and political attention at the end of the 20th century, the so-called persistent organic pollutants (POPs). POPs are organic compounds

that have prolonged half-lives due to strongly retarded degrading processes (Jones & De Vogt, 1999). The publication of the book Silent Spring by R. Carson in 1962 drew already attention to some of these highly chlorinated substrates in the environment that are traceable in the environment for decades. Over time, studies provided enough evidence that POPs also physically harm the biota (Eisler & Edmunds, 1966; Liu et al., 2008; Constantini et al., 2014). Some of them demonstrably act like endocrine substances, disrupting animals' reproduction. Others, in turn, appear to inhibit important steps during development, act as carcinogens, or affect the function of the immune system. To some extent, all POPs have an acute or delayed impact on health of living organisms (Johnson et al., 2013). Furthermore, international concern arose by finding POPs' ability to migrate long distances through the atmosphere (Beyer et al., 2000). POPs can therefore be detected far from their source regions, where influences from agriculture and industry are unexpected. Consequently, the impact of POP release is expanding from a local, to a regional and finally to a global level. In the year 2004, an international agreement came into force: the production and use of 12 chemical classes was globally banned. The prohibition includes amongst others polychlorinated biphenyls, brominated flame retardants, and several pesticides such as DDT, endrin, and endosulfan (United Nations Environment Program, 2017). Despite the adoption of the Stockholm Convention in 2004, the chemical compounds are still detectable in nature (Pribylova et al., 2012; Werner & Hitzfeld, 2012; Hung et al., 2016).

The POPs listed in the Stockholm Convention can be divided into two subgroups: hydrophilic and hydrophobic or lipophilic (Muir & Lohmann, 2013). Depending on the respective chemical behavior, POPs slowly accumulate in organisms or get washed out right after their uptake. Potential inanimate sinks (and sources) for persistent pollutants are air, water and soils (Lohmann et al., 2007). Atmospherically transported POPs reach the earth surface via precipitation. This leads to severe pollution of ecosystems with high precipitation quantities, like mountain regions in temperate zones. Thus, the deposit rate of atmospherically transported POPs on a remote mountain can be higher than that of the valley floor (Wania & Mackay, 1996). Nonetheless, only little research so far has dealt with the impact of increased POP burden on mountain regions. Having reached the ground, the chemical compounds incorporate into the soil matrix. The extent of the incorporation depends on the sorption capacity of the soil (Ren et al., 2018), which builds upon the composition and the amount of organic matter (OM). Large fractions stay bound and remain unavailable to flora and fauna (Ehlers & Loibner, 2006). After depletion of OM, there is a certain risk that these sorbed chemicals get released into the ambient environment and subsequently can be taken up through the food chain. Thus, the bioavailability is premised on the reversibility of binding pollutants. During hot summer months, POPs easily evaporate from the soil and saturate the

overlying layer with toxic substances that are taken up by above ground-living organisms, like plants and foraging pollinators (Sari et al., 2020).

There are numerous ways for biota to get in touch with POPs, and it is hard to evaluate the route of uptake. For example, plants can soak up water-soluble POPs via water adsorption from contaminated soils (Kacálková & Tlustoš, 2011), whereas another part of contamination occurs above the ground through aerial deposition onto the surface of leaves (Franzaring & Van Der Eerden, 2000). This combination of aboveground and subterraneous contamination is one reason why flowers were found to be more heavily polluted than soils. Native wild bees make up a considerable part of flowering visitors, and are thus indispensable pollinators (Winfree, 2010). By collecting pollen and nectar, bees are highly exposed to these POPs and pass it on to all life stages (Roszko et al., 2016). Once ingested, particularly lipophilic POPs become embedded into the fatty tissue (Jones and de Voogt, 1999). Many studies examine the effects of bioaccumulation and biomagnification in different trophic levels along a food chain (Jones & de Voogt, 1999; Mackay & Fraser, 2000; Kelly et al., 2007). Altered gene expression is also a probable outcome of exposure to mixtures of POPs in the environment of vertebrates (Lyche et al., 2013). Disorders at delicate developmental stages ultimately cause phenotypic changes (Foekema et al., 2012) like modifications in shape, size and fluctuating asymmetry (Jenssen et al., 2009). Fluctuating asymmetry (FA) is thus a valid fitness proxy and a widely used indicator to monitor environmental and genomic stress (Klingenberg, 2003; Beasley et al., 2013). However, most research so far has been conducted in the lab, where POP treatments were performed under controlled conditions. A pilot study conducted in 2018 by Struck et al. (in prep.) set the required methodological framework to tackle the challenge of finding potential links between FA and unknown a-priori POP burden in insects in high-elevation ecosystems.

Bees in general are widely used to monitor POPs and heavy metals (Kevan, 1999; Al-Alam et al., 2019). Bumblebees are cold-adapted species climbing up high-mountain environments. Due to their ability to generate heat (Heinrich, 1975), bumblebees are the most efficient pollinators in alpine ecosystems (Bingham & Ranker, 2000). In our view, they are the biomonitoring organism of choice to provide information about the alpine airborne pollution levels. The social structure of *Bombus* spp. allows the collection of enough biomass for analysis without jeopardizing the colonies' survival when compared with other, solitary wild bees. Due to the short lifetime of bumblebees, the quantity of POPs detected within an individual's body does not represent the extent of bioaccumulation over years. Furthermore, the foraging range extends 0.5 to 1.5 km around the colony (Knight et al., 2005; Osborne et al., 2008). Thus, bumblebees offer a snapshot of the actual burden within their foraging area.

The first goal of this study was to evaluate the pollution load in two bumblebee species, a mountain specialist and an altitudinal generalist, at two subalpine meadows above the tree line by measuring POP and mercury burden in pooled samples. Meteorological data showed a dissimilar extent of pollution in the ambient air concentration comparing the two locations (Kirchner et al., 2020). Hence, we assumed that this difference in contamination is also detectable within the local bumblebee fauna. The chemical analyses in our study targeted POPs listed in the Stockholm Convention and the heavy metal mercury, which possesses similar characteristics concerning the toxicity to biota, the persistence in the environment, and the accumulation behavior.

The second objective addressed implications regarding the pollution load by means of geometric morphometrics and population genetics. If genetical factors, like inbreeding, can be excluded, the observation of left-right asymmetry in wings is probably the product of environmental stressors. If so, we aimed at revealing to what extent POPs play a role within the spectrum of environmental stressors acting on Alpine bumblebees.

Mountains are characterized by fast changing weather periods, short growth periods, high radiation exposure, low air pressure and environmental conditions changing rapidly over short distances along an elevation gradient. With pollution and climate change joining in, alpine inhabitants have to cope with multiple stressors to survive and reproduce. Our knowledge on the interplay of these stressors still comprises large gaps (Kaunisto et al., 2016). The present study is intended to contribute to a better understanding to mitigate the fast-ongoing insect decline, since besides Lepidoptera and Coleoptera, Hymenoptera is the taxa most affected insect taxa (Sánchez-Bayo & Wyckhuys, 2019). With an adequate strategy to ensure pollinators' survival, not only will relevant ecosystem services be maintained, but also human well-being (Potts et al., 2016).

2 Materials & Methods

2.1 Study area & species profiles

The study was carried out close to the two meteorological stations, Schneefernerhaus at Zugspitze (Germany) and Sonnblick Observatory at Hoher Sonnblick (Austria), where atmospheric POP concentrations have continuously been recorded since 2005 (Freier et al., 2019). The sampling sites were comparable subalpine meadows above the tree line at elevations ranging from 1'700 and 1'900 m above sea level and were characterized by knee timber, dwarf shrubs and typical subalpine turf. The main floral source for local pollinators were alpine roses (*Rhododendron* spp.) and blueberry bushes (*Vaccinium* spp.).

In the framework of the present study, the focus lied on the two most abundant bumblebee species of both the Zugspitze and the Hoher Sonnblick, because of the high amount of biomass needed to conduct chemical analyses (Struck et al., in prep.): *Bombus pratorum* is a ubiquitous species covering many elevation levels. The Early bumblebee, as this species is also called, nests mainly hypogeous beneath scrub and mosses. *Bombus wurflenii,* in contrast, is a primarily mountainous species, living close to forests and nearby (sub-)alpine pastures. Its predominant endogenous nesting habit exposes the individuals, beside airborne contamination, also to pollution in the soil that accumulated through the years.

2.2 Sampling

To keep the sampling pressure on the colonies low, bumblebees were sampled over a period from beginning of July to the middle of August 2019. Only worker bumblebees were caught. We obtained a permission from the Nationalpark Hohe Tauern to collect individuals of the genus *Bombus* around the Hoher Sonnblick, excluding vulnerable species such as *B. alpinus* (see Appendix 1 & 2).

To avoid contamination during the manual sampling procedures, the bumblebees were caught individually by using cleaned Duran® borosilicate glass flasks. After catching, the bees were directly cooled down in the field and instantly euthanized in a dry shipper containing liquid nitrogen. In the laboratory, the individuals were wrapped into aluminum foil, put separately in glass tubes and stored in a freezer (-20 °C) prior to further steps. Sampling, species identification and sample processing followed the clean handling techniques established by Struck et al. (in prep.) to

minimize contamination of the samples. All work was done on ice packs to maintain the cooling chain.

2.3 Sample preparation & chemical analyses

Prior to chemical analysis to measure the POP concentrations in the insect bodies, bumblebees were prepared accordingly. Both pairs of wings (fore- and hindwing) and three legs from each individual were pinched off with cleaned forceps and stored separately in 96% ethanol. The remaining bodies were packed together into pool samples, sorted by species and sampling location (Hoher Sonnblick & Zugspitze), i.e., in four populations: *Bombus pratorum* Hoher Sonnblick (BpS), *B. pratorum* Zugspitze (BpZ), *B. wurflenii* Hoher Sonnblick (BwS), *B. wurflenii* Zugspitze (BwZ). Each pool sample was weighed, and the respective number of individuals counted.

Chemical analyses were done at an external lab in Vienna, run by the Environment Agency Austria. The pool samples were homogenized and lyophilized before entering the atomic fluorescence spectroscopy (AFS) and gas chromatography/high-resolution mass spectrometry (GC/HRMS). In this study, mercury (Hg) and a total of 77 substances included in the Stockholm Convention were analyzed (all of them listed in Appendix 3): Brominated flame retardants (BFR), polychlorinated biphenyls (PCB), polyfluorinated chemicals (PFC) and organochlorinated pesticides (OCP) including, amongst others, DDT and its metabolites, hexachloro-cyclohexanes and various chlordanes.

2.4 Microsatellite genotyping & population genetics

Two legs of each bumblebee were ground, extracted and eluted using the QIAGEN DNeasy® Blood and Tissue Kit (Qiagen, USA) following the instructions by the manufacturer. The extracted DNA was then temporarily stored in the refrigerator at 4 °C and processed immediately.

Each bumblebee species was genotyped at nine loci, either developed by Estoup et al. (1993) or Funk et al. (2006). The primers were originally designed for *B. lucorum*, *B. terrestris*, or *B. ternarius*. Funk et al. checked for cross-amplification in different *Bombus* species, inter alia, *B. wurflenii*. All species were genotyped at loci BT08, BL11, BT11, BTERN01, BTERN02. *Bombus pratorum* was additionally genotyped at B96, B100, B124 and B126 and *B. wurflenii* at BT10, BT28, BT30, BL13. For the amplification of all loci, a polymerase chain reaction (PCR)-

formulation was prepared with 0.5 µl of the bumblebee sample DNA and 4.5 µl of a reaction dissolution consisting of: 3.75 µl Milli-Q® water, 1 µl 5X OneTaq® Quick-Load® Reaction Buffer, 0.1 µl dNTPs (10mM), 0.1 µl forward primer (10 µM), 0.1 µl labeled forward primer (10 µM), 0.1 µl reverse primer (10 µM) and 0.025 µl OneTaq® Quick-Load® DNA Polymerase. Amplification of DNA strands was done with a forward primer containing a M13-tail at the 5'-end and the universal M13 forward primer, labeled with universal fluorescent dyes HEX, NED, PET and FAM. Cycling conditions varied among the two species with regard to the denaturing and the first cycling steps: The initial denaturing step of 3 min was at a temperature of 94 °C for *B. pratorum* and at 95 °C for *B. wurflenii*. Denaturing was followed by 35 cycles that started for 30 *s* at 92 °C for *B. wurflenii* and 94 °C for *B. pratorum,* then moved on for another 30 s at the optimal annealing temperature for the relevant locus (Estoup et al., 1993; Funk et al., 2006), and finally closed with 30 s at 72 °C. At the end of the very last cycle, the final elongation step was extended to 10 min at 72 °C. The occurrence of PCR-products was verified by running an agarose gel electrophoresis. The amplicons were sent to a commercial supplier, the Comprehensive Cancer Center DNA Sequencing & Genotyping Facility at the University of Chicago (USA), for Sanger sequencing.

The sequenced DNA fragments were visualized with GeneMarker® v.3.0.1 (SoftGenetics, State College, PA, U.S.A.), using the GS600 LIZ size standard (Applied Biosystems). Apparent alleles were scored manually.

With the help of the package «genepop» (Rousset, 2008) in RStudio v.3.3.3 (RStudio Team, 2021), the selected markers were examined in terms of suitability for further analysis. The linkage-disequilibrium (LD) compared pairwise all alleles over the selected loci to check for independent occurrence. We further tested for deviations from the Hardy-Weinberg equilibrium (HWE) to reveal evolutional pressure acting on the populations. Through a subsequent Bonferroni-Holm correction we could adjust for multiple comparisons and preclude false-positive LD- and HWE-outputs. We also considered potential amplification failures by means of microsatellite null allele testing. Based on the observed heterozygosity (H_o), the numbers of expected heterozygotes (H_e) under the HWE were generated using the Levene's correction. Hierarchical F-statistics were computed by applying the methods of Weir & Cockerham (1984).

Different inbreeding coefficients are in use to quantify potential inbreeding in populations. We decided to use the multilocus heterozygosity (MLH) estimate as a suitable approach (Hansson, 2010). MLH is calculated as the total number of heterozygous loci within one individual divided by the number of loci typed in the same individual. The corresponding calculation was performed with the «inbreedR» package (Stoffel et al., 2016). Statistical tests were implemented in RStudio v.3.3.3 to check for differences among the two species and populations.

2.5 Landmark based geometric morphometrics

Methods concerning geometric morphometrics were slightly adapted from Struck et al. (in prep). All specimens were photographed and landmarked by one person (S. Gurten) to exclude experimenter bias.

Each detached wing was evenly placed in between two microscope slides and fixed with clamps to get a planar capture. Pictures were taken through a Leica Z6 APO macroscope (Leica Microsystems, Wetzlar, Germany) with a built-in Leica DFC 420 camera. Image processing was done with LAS (v3.6.0). All four wings of a bumblebee individual were shot twice, once from the top and once from beneath. Half of the image set was mirrored so that all photos faced the same direction to prevent biases in landmark setting. These pictures, saved as .jpeg-files, were converted into .tps-files using tpsUtil v1.76 (Rohlf, 2015). Landmarking was manually done via tpsDig2 v2.31 (Rohlf, 2015) by positioning the landmarks at vein intersections as depicted in Aytekin et al. (2007), resulting in 20 landmarks for the forewings and six for the hindwings.

The uniformity of setting landmarks is crucial to rely on the results. Overall, there are three possible sources of variation: 1.) Biological variation caused by development instabilities, 2.) methodological distortion through photographical settings (imaging error), and 3.) methodological errors due to e.g. too much leeway in placing landmarks (digitizing error). Aiming at detecting biological variation, if present, we wanted to minimize the latter two. A pilot study was created to determine the proportions of the variation. Per sampling location, 20 bumblebee individuals from *B. pratorum* were selected and photographed a second time, after having removed the fixed wing and replaced it in order to consider a positioning bias. These 40 individuals were digitized twice.

The generated dataset was then analyzed with the software MorphoJ v1.07a (Klingenberg, 2011). A Procrustes fit was carried out to remove size and shape variation due to differences in scale and orientation of raw landmark data to finally create aligned wing coordinates. Procrustes analyses of variance (ANOVA) were conducted thereafter to test for differences in centroid size and shape among individuals. Wing pairs with a missing photograph or incomplete landmarking of the counterpart wing side were excluded for analysis on individual level. To measure the FA within an individual bumblebee, we exported the generated individual FA scores for wing shape asymmetry. In addition, the FA scores for wing size asymmetry were calculated manually, based on the FA index 2 formula (Padró et al., 2014).

2.6 Modeling: Uncovering the roots of wing asymmetry

To reveal potential links between genetics, pollutant burden and the manifestation of fluctuating wing asymmetry, different statistical tests and models were conducted in RStudio v.3.3.3. Besides the basic statistics, the following packages were used: «lmtest» (Zeileis & Hothorn, 2002), «vegan» (Oksanen et al., 2017), «ggplot2» (Wickham, 2016), «performance-Estimation» (Torgo, 2014).

Typal multiple linear models (MLM) were used to explore the relationship between the dependent variables, FA index 2 scores of either fore- or hindwing size or shape, and the two independent variables, inbreeding coefficient MLH and the measured POPs. Rows that contained missing entries (n.a.) were excluded from linear regression analysis. Where residual analysis showed poor model fits, a logarithmic transformation was done to achieve a reasonable normal distribution of the residuals. To get statistical support for normal distribution and heteroskedasticity, Shapiro-Wilk (Royston, 1982) and Breusch-Pagan (1979) tests were performed, respectively.

To take chemical measurement inaccuracy into consideration, Monte Carlo simulations (MCS) were run to test for model output robustness. According to the Environment Agency Austria (personal communication), deviations of +/-30% for POP and +/-13% for mercury values could be expected. Simulations including these inaccuracy extremes over six groups were done with the following numbers of MCS-runs: 20, 100, 200, 1000, 2000, 10,000. Both the median p-value and the median R^2-value from model outputs were graphically depicted for each simulation group (see example in Appendix 4). The amplitude of median variation over the course of simulation operated as indication where to finally stop: After 1000 MonteCarlo-runs most model output values stabilized. As soon as a model's median p-value remained significant after 1000 MonteCarlo-runs, its origin result could be accepted and further interpreted. Otherwise, the original model was rejected.

3 Results

3.1 Bumblebee collection

In total, we collected 209 worker bees of *Bombus pratorum* and 122 of *B. wurflenii* (Table 1). The remaining 571 specimens caught (see Box 1) were processed in the framework of the project «protectAlps» and will not be further discussed in this study.

Table 1. *Total number of specimens caught split into species and sampling location.*

species	sampling location	population	# individuals
Bombus pratorum	Hoher Sonnblick	BpS	126
	Zugspitze	BpZ	83
Bombus wurflenii	Hoher Sonnblick	BwS	51
	Zugspitze	BwZ	71
Total			331

3.2 POP and mercury burden

Overall, 35 out of 77 evaluated POPs could be detected (Appendix 3), some of them actually at the limit of quantification. For ten substances thereof (p-TBX, PBEB, PBB153, syn-DP, BDE 28, BDE 66, BDE 77, BDE 118, BDE 139, BDE 181), the measurement values laid in-between the quantification limit and the limit of detection. All the other remaining 25 detected substances (depicted in Fig. 1A and Fig. 1B) could be quantified and showed contrasty values, either at sampling location and/or species level.

OCPs and PFCs were not detectable in our bumblebee samples. Of the verified POPs, only one pool sample could not be analyzed accordingly, namely DBDPE in BpS (*B. pratorum*, Sonnblick): Matrix effects affected the interpretation of the chromatogram's output. Every POP showed higher to marginally smaller (i.e., anti-DP and BDE 207 in *B. pratorum*) measurement values at Hoher Sonnblick, except for DBDPE. No POP was unique for a sampling location or species.

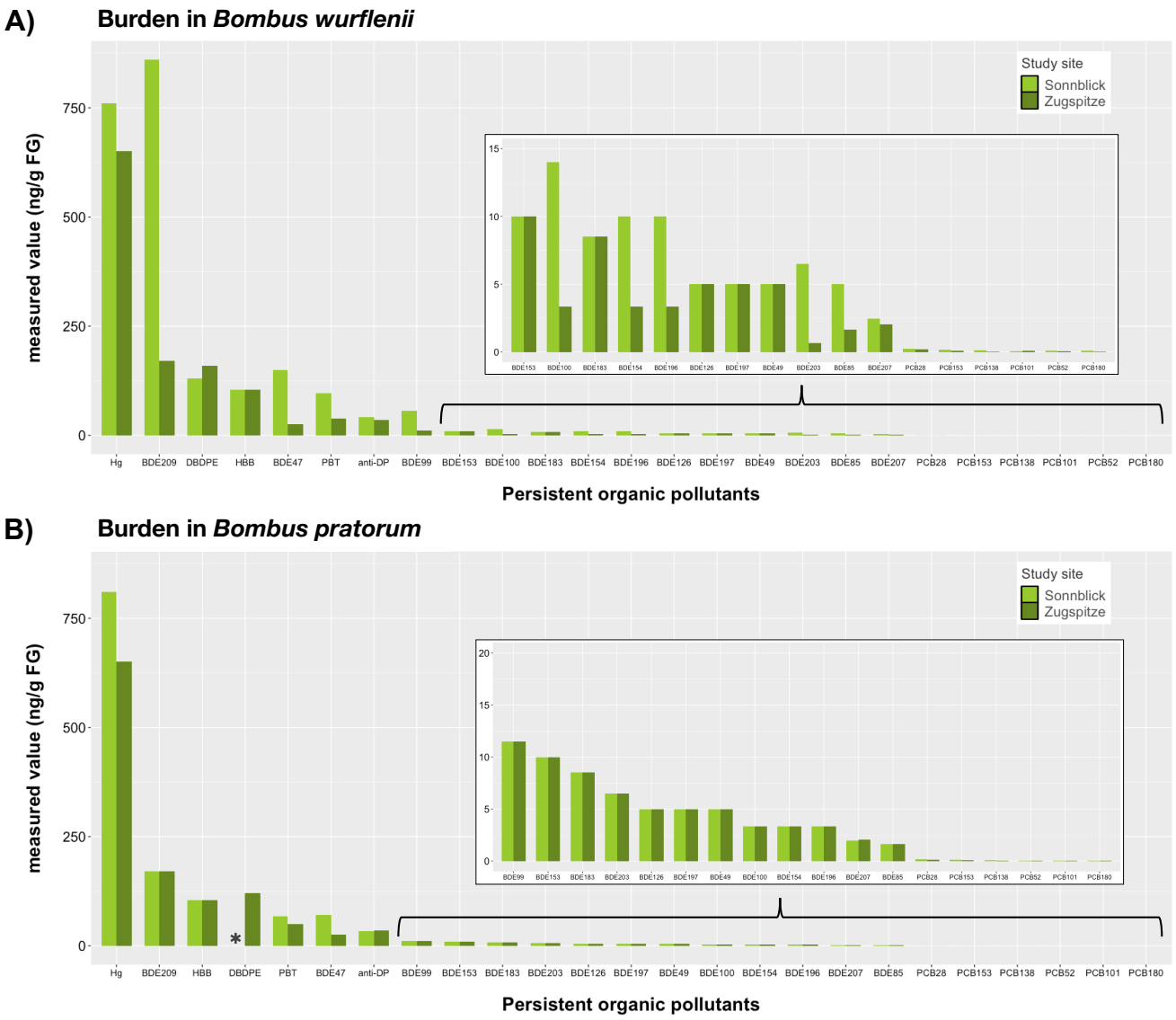

Figure 1. *Persistent organic pollutants concentrations in (ng/g) wet weight (FG), listed in descending order, according to their amount detected in the respective samples. Only the actually measured values are illustrated, without considering measurement inaccuracies. The sampling sites are indicated in contrasting green shades. The zoom-in extract includes all measured values below 15 ng/g FG.* ***A)*** *Burden measured in Bombus wurflenii.* ***B)*** *Burden in B. pratorum.* ***(*)*** *stands for no value available (n.a.).*

Both populations at the Zugspitze, BwZ and BpZ, demonstrated the same amount of mercury pollution (650 ng per 1.0 g wet weight). In contrast, the mercury loads within the bumblebee populations at Hoher Sonnblick, BwS and BpS, species-specifically differed.

3.3 Population genetics

Microsatellite data validation. For *B. wurflenii*, all nine microsatellite loci could be scored consistently across both populations. In *B. pratorum*, one population failed to amplify at locus BTERN01 and was excluded from further analysis in consequence of a broad allelic dropout at the Zugspitze population (Table 2). For the loci B100 and B126, a null allele frequency of > 10 % had

been estimated and represented a knock-out criterion in combination with significant deviations from the HWE. BL13 in *B. wurflenii* constituted another marker for exclusion by exhibiting only one allele in the population BwZ and just a few in BwS, too.

Table 2. *Final scoring expressed in numbers. (* #) represent the total number of genotyped individuals. Red colored entries indicate excluded loci. Abbreviations pose the respective population: BpZ = Bombus pratorum, Zugspitze; BpS = B. pratorum, Hoher Sonnblick; BwZ = B. wurflenii, Zugspitze; BwS = B. wurflenii, Hoher Sonnblick.*

Locus	**Label**	**# BwZ** (* 71)	**# BwS** (* 51)	**Locus**	**Label**	**# BpZ** (* 83)	**# BpS** (* 126)
BL11	PET	58	46	B96	FAM	71	96
BT30	HEX	41	30	B100	HEX	46	75
BL13	FAM	54	29	B124	NED	58	98
BT10	HEX	35	28	B126	PET	61	95
BT28	FAM	27	24	BT11	NED	63	53
BT11	NED	23	19	BL11	PET	69	96
BTERN01	NED	56	41	BTERN01	NED	10	81
BTERN02	PET	26	17	BTERN02	PET	42	76
BT08	FAM	57	27	BT08	FAM	40	76

Apart from one locus combination (i.e., BL11 & BT11 in the BpZ population), no other locus combination was significantly linked after a sequential Bonferroni-Holm correction with a local $\alpha = 0.05$. In terms of Hardy-Weinberg, three out of eight loci deviated significantly from equilibrium in *B. wurflenii* (BL11, BTERN01, BTERN02), but all of them only in the Zugspitze population (BwZ). Among the remaining six loci in *B. pratorum*, two markers in the population BpS (BT08, BTERN02) and three in BpZ (B96, BL11, BTERN02) still displayed significance after Bonferroni-Holm correction.

Heterozygosity. All remaining loci were polymorphic, with two to 23 alleles and 10 to 25 alleles per locus for *B. wurflenii* (Table 3) and *B. pratorum* (Table 4), respectively. Mean expected and observed heterozygosities across loci in BwS were 0.80 and 0.82, respectively, with five loci depicting a higher H_o than H_e and in BwZ 0.79 and 0.75, respectively, with four loci showing higher H_o than H_e. The mean expected and observed heterozygosities in *B. pratorum* ranged from 0.84 to 0.77 in BpS, respectively and 0.81 to 0.83, respectively, in BpZ. All loci of the Sonnblick's population (BpS) indicated lower H_o than H_e, while at the Zugspitze (BpZ) three loci showed higher H_o than H_e.

Table 3. *Estimates of genetic diversity based on eight microsatellite loci in Bombus wurflenii, including allelic richness (N_A), expected (H_e) and observed heterozygosity (H_o) and inbreeding coefficient F_{IS}. Abbreviations pose the respective population: BpZ = B. pratorum, Zugspitze; BpS = B. pratorum, Hoher Sonnblick; BwZ = B. wurflenii, Zugspitze; BwS = B. wurflenii, Hoher Sonnblick.*

	Hoher Sonnblick (BwS)				Zugspitze (BwZ)			
Locus	**N_A**	**H_e**	**H_o**	**F_{IS}**	**N_A**	**H_e**	**H_o**	**F_{IS}**
BL11	20	0.9242	0.9048	0.0214	19	0.9139	0.9259	-0.0132
BT30	7	0.6684	0.7000	-0.0482	7	0.5649	0.6341	-0.1243
BT10	17	0.9396	1.0000	-0.0655	21	0.9449	0.8857	0.0635
BT28	2	0.3112	0.3750	-0.2105	2	0.2830	0.3333	-0.1818
BT11	7	0.8264	0.8421	-0.0195	8	0.8270	0.6956	0.1619
BTERN01	19	0.9205	0.9024	0.0199	23	0.9385	0.9464	-0.0085
BTERN02	17	0.8823	1.0000	-0.0667	19	0.9351	0.8077	0.1386
BT08	22	0.9518	0.8148	0.1463	19	0.9124	0.7894	0.1358
Overall	13.8750	0.8031	0.8174	-0.0279	14.7500	0.7900	0.7523	0.0215

Table 4. *Estimates of genetic diversity based on eight microsatellite loci in Bombus pratorum, including allelic richness (N_A), expected (H_e) and observed heterozygosity (H_o) and inbreeding coefficient F_{IS}. Abbreviations pose the respective population: BpZ = B. pratorum, Zugspitze; BpS = B. pratorum, Hoher Sonnblick; BwZ = B. wurflenii, Zugspitze; BwS = B. wurflenii, Hoher Sonnblick.*

	Hoher Sonnblick (BpS)				Zugspitze (BpZ)			
Locus	**N_A**	**H_e**	**H_o**	**F_{IS}**	**N_A**	**H_e**	**H_o**	**F_{IS}**
B96	16	0.9015	0.8316	0.0780	16	0.8873	0.8194	0.0770
BL11	25	0.9198	0.8958	0.0745	19	0.6933	0.9325	-0.0211
B124	10	0.7571	0.7010	0.0262	10	0.7631	0.6610	0.1348
BT08	13	0.8229	0.7237	0.1213	10	0.8474	0.8500	-0.0030
BT11	17	0.8428	0.7925	0.0602	16	0.8123	0.8412	-0.0359
BTERN02	25	0.8030	0.6842	0.1488	24	0.8930	0.8809	0.0137
Overall	17.6667	0.8412	0.7715	0.0848	15.8333	0.8161	0.8308	0.0276

Inbreeding coefficient (F_{IS}). The F_{IS}-values over all loci laid close to zero, depicting almost ideal populations (Table 3 & 4). The population BwS displayed negligible outbreeding, all the others (BwZ, BpS, BpZ) marginal inbreeding, if at all.

Multilocus heterozygosity (MLH). MLH values were consistently high in both species (Figure 2). More than half of all individuals genotyped were characterized as having 65% of all loci (or more) heterozygous. No difference in the individual MLH values of *B. wurflenii* could be detected when comparing both locations (p = 0.3622). However, there was a difference in the MLH means (p = 0.0457) between the two *B. pratorum* populations (0.7748, Zugspitze & 0.8318, Hoher Sonnblick; Fig. 2).

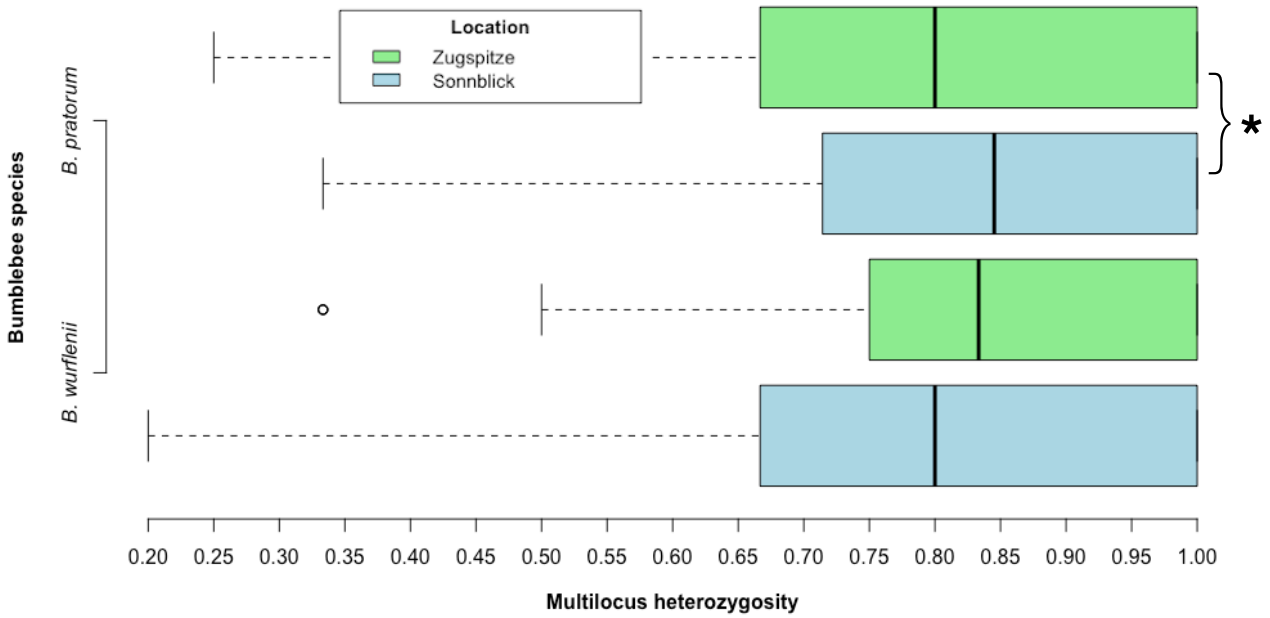

Figure 2. *Multilocus heterozygosity in both species. (*) marks significant differences between two MLH mean values. The boxes represent the interquartile range from the first to the third quartile, the lines across the boxes indicate the median, the whiskers represent the quartiles±(1.5×the interquartile distance) and the open circles indicate outliers.*

3.4 Fluctuating asymmetry

Digitizing and imaging error. The examination of the pilot data revealed no statistically significant errors concerning digitizing or imaging, neither for fore- nor for hindwings. The variation, indicated as F-values and the mean sum of squares, explained by the image setting and the landmarking manner was consistently lower than that established by differences among individuals or FA-values (Appendix 5).

Table 5. *Results of the Procrustes analysis of variance for wing shape comparing both sides of Bombus pratorum. SS, sum of squares; MS, mean sum of squares; df, degrees of freedom; F, variation; p, p-value. Populations, where fluctuating asymmetry explained more variation than other classifiers, are marked (*), with relevant F-values printed in bold.*

wing	location	classifier	SS	MS	df	F	p
forewing shape	Zugspitze	Individual	0.1050	0.00004354	2412	6.80	< 0.0001
		Fluctuating asymmetry	0.1544	0.00011218	2412	6.04	< 0.0001
		Imaging error	0.0044	0.00000106	4176		
	Hoher Sonnblick	Individual	0.1964	0.00005454	3600	2.26	< 0.0001
		Fluctuating asymmetry	0.0870	0.00002417	3600	1.18	< 0.0001
		Imaging error	0.1329	0.00002051	6480		
hindwing shape	Zugspitze	Individual	0.1853	0.00030878	600	7.82	< 0.0001
		Fluctuating asymmetry	0.0237	0.00003948	600	4.79	< 0.0001
		Imaging error	0.0009	0.00000825	1064		
	Hoher Sonnblick *	Individual	0.1604	0.00019278	832	4.05	< 0.0001
		Fluctuating asymmetry	0.0396	0.00004758	832	6.19	< 0.0001
		Imaging error	0.0119	0.00000768	1544		

Table 6. *Procrustes analysis of variance testing for differences in wing shape FA in Bombus wurflenii. SS, sum of squares; MS, mean sum of squares; df, degrees of freedom; F, variation; p, p-value. Populations, where fluctuating asymmetry explained more variation than other classifiers, are marked (*), with relevant F-values printed in bold.*

wing	location	classifier	SS	MS	df	F	p
forewing shape	Zugspitze	Individual	0.0879	0.00003756	2340	7.53	< 0.0001
		Fluctuating asymmetry	0.0117	0.00000499	2340	5.45	< 0.0001
		Imaging error	0.0040	0.00000092	4392		
	Hoher Sonnblick *	Individual	0.0629	0.00003968	1584	5.49	< 0.0001
		Fluctuating asymmetry	0.0114	0.00000722	1584	8.45	< 0.0001
		Imaging error	0.0026	0.00000086	2988		
hindwing shape	Zugspitze *	Individual	0.0949	0.00017449	544	5.09	< 0.0001
		Fluctuating asymmetry	0.0186	0.00003427	544	5.78	< 0.0001
		Imaging error	0.0061	0.00000593	1032		
	Hoher Sonnblick *	Individual	0.0505	0.00014017	360	4.29	< 0.0001
		Fluctuating asymmetry	0.0118	0.00013840	360	4.48	< 0.0001
		Imaging error	0.0051	0.00000730	704		

Overall fluctuating asymmetry. We found consistently significant results in both species ($p < 0.0001$), meaning that there were substantial differences between left and right wings in both size and shape. The evaluation of the wing size FA (results depicted in Box 2: Table 2) generally showed higher F-values than those of wing shape FA (Table 5 & 6). Wing size FA F-values were high, accounting for multiple times the variation of wing sizes among individual bumblebees (see Table 2 in Box 2). Shape FA was comparable with the inter-individual variation. Within many populations, FA of fore- and hindwings explained more variation than individuals (marked with a star in Table 5 & 6, and in Table 2 in Box 2).

Individual FA scores between study sites. When comparing both sampling sites at species level (Fig. 3), significant differences in individual FA scores were only found in the forewing shape of *B. wurflenii* ($p = 4.35 \times 10^{-06}$). With respect to the hindwing shape and the fore- and hindwing size, we did not find any significant difference in FA indices between the two study sites.

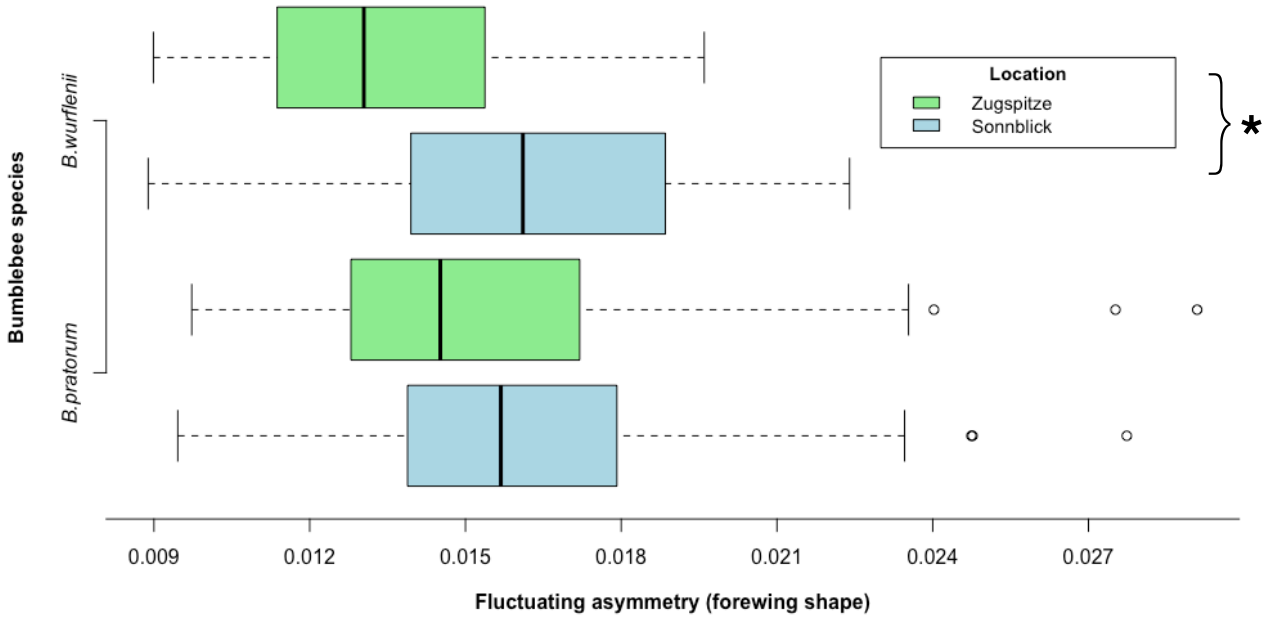

Figure 3. *Fluctuating asymmetry (FA) scores in the forewing shape of both species at both sampling sites. The asterisk (*) represents significances in difference between two FA score mean values. The boxes represent the interquartile range from the first to the third quartile, the lines across the boxes indicate the median, the whiskers represent the quartiles±(1.5×the interquartile distance) and the open circles indicate outliers.*

3.5 Correlation between genetics, pollution load and FA

The forewing shape FA scores in *Bombus wurflenii* were significantly linked to virtually all POPs (Table 7). After MCS, some significance disappeared (marked as (***) in Table 7). Genetics

(MLH) was negatively related with the FA in forewing shape; i.e., with increasing MLH values, the forewing shape FA scores decrease. All POPs, except for PCB101 and DBDPE, had a positive effect on the forewing shape FA. Genetics and POPs together explained around 20 % (multiple R^2) of the variation in left-right forewing shape asymmetry.

There was no significant correlation across both species between the wing size FA scores, the MLH value and the POP load. We also found no correlation by replacing size by shape scores, except for the forewing in *B. wurflenii*.

Table 7. *Results of multiple linear models (z = ax + by +c); with ‚z' = forewing shape FA score; ‚x' = MLH value; ‚y' = POP; ‚c' as intercept. *** means p < 0.05.*

Linear model (z ~ ax + by + c)	**a (slope MLH)**	**b (slope pollutant)**	**No. of models significant out of 1'000 MC runs**	**significance (before MC)**
forewing shape FA ~ MLH + Hg	-0.0014773	2.497×10^{-05}	592	***
forewing shape FA ~ MLH + PCB28	-0.0014773	0.137315	53	(***)
forewing shape FA ~ MLH + PCB52	-0.0014773	2.746293	16	(***)
forewing shape FA ~ MLH + PCB101	-0.0014773	-0.274629	83	(***)
forewing shape FA ~ MLH + PCB138	-0.0014773	0.059702	769	***
forewing shape FA ~ MLH + PCB153	-0.0014773	0.036617	799	***
forewing shape FA ~ MLH + PCB180	-0.0014773	0.072271	815	***
forewing shape FA ~ MLH + PBT	-0.0014773	4.655×10^{-05}	724	***
forewing shape FA ~ MLH + `anti-DP`	-0.0014773	1.962×10^{-04}	191	(***)
forewing shape FA ~ MLH + DBDPE	-0.0014773	-9.154×10^{-05}	218	(***)
forewing shape FA ~ MLH + BDE47	-0.0014773	2.746×10^{-05}	641	***
forewing shape FA ~ MLH + BDE85	-0.0014773	0.0016155	665	***
forewing shape FA ~ MLH + BDE99	-0.0014773	8.322×10^{-05}	708	***
forewing shape FA ~ MLH + BDE100	-0.0014773	3.762×10^{-04}	708	***
forewing shape FA ~ MLH + BDE154	-0.0014773	0.0008322	677	***
forewing shape FA ~ MLH + BDE196	-0.0014773	0.0008322	677	***
forewing shape FA ~ MLH + BDE207	-0.0014773	0.0034329	189	(***)
forewing shape FA ~ MLH + BDE209	-0.0014773	5.281×10^{-06}	716	***

4 Discussion

Pollution burden. Persistent (organic) pollutants determined by the environment research stations in the mountainous study areas (Freier et al., 2019) were detectable in Alpine bumblebees, with the exception of OCPs and PFCs. These findings support the hypothesis that the atmospherically measured POP & Hg load is reflected in the local fauna, despite the actual source being temporally and spatially far away. However, the number of substances measured in the insect samples does not necessarily correlate with the degree of the respective detrimental impact. The compounds' mode of action largely remains understudied in insects (Hierlmeier et al., in prep).

Differences within substances were evident both on mountain and on species level. In the pre-alps, the annual precipitation rates are higher than in the central mountain range; therefore, the Zugspitze environment is thought to be higher contaminated than the environment around the Hoher Sonnblick (Jakobi et al., 2015). Unexpectedly, this topological effect was not reflected in the POP and mercury concentrations of the investigated bumblebee bodies. Both bumblebee species on average showed higher pollution burden at Hoher Sonnblick compared with the Zugspitze. From a quantitative point of view, the population BwS was highest contaminated.

OPCs comprise widespread pesticides in history. Nevertheless, they were not detectable within our bumblebee samples. PFCs have not been detected, although they have been used over years in a myriad of consumer products. Actually, main sources of PFC are contaminated water and sewage sludge (Guo et al., 2019). This partial water solubility of PFCs is also reflected in the high number of publications dealing with aquatic ecosystems (Giesy & Kannan, 2001). Because PFC exhibits a high affinity to polymers (Fernandez-Sanjuan et al., 2010), it is assumed to be quite inaccessible for terrestrial biota when integrated into soil. Plant roots, in turn, only assimilate substances with a sufficient low capacity for sorption (Prevedouros et al., 2003). Substances, accumulated by plants, are then ingested by the bumblebees.

Mercury was the only xenobiotic metal investigated. It is released from industry and traffic and is among the most toxic heavy metal contaminants (Braeckman & Raes, 1999). The concentrations measured show highly alarming values; they are comparable with ants and mantis caught in Huludao City, an important chemical industry area in the Northeast of China (Zhang et al., 2012). However, ants and mantis are omni- and carnivorous species, respectively and thus belong to a higher trophic level than bumblebees. With higher trophic level, the pollution content can be measured according to the process of bioaccumulation and biomagnification along a food chain (Tsui et al., 2009; Jardine et al., 2013; Jones et al., 2013; Rowse et al., 2014). Consequently, we

assume that omnivores and carnivores at the Zugspitze and the Hoher Sonnblick bear even higher heavy metal burdens.

Pollution implications. There is undoubted evidence that POPs and heavy metals in plants and animals affect different morphological traits in their size and/or shape (Jenssen et al., 2010; Neustupa & Woodard, 2019; Quina et al., 2019). In our study, we were able to confirm the relationship between POP & mercury burden and the fluctuating asymmetry in bumblebee wings: Apart from a few exceptions, we found significant positive correlations for POPs or mercury and FA in *B. wurflenii*; this in turn means that the higher the POP load within a sample, the larger the measured wing asymmetry in the respective individual. Around 20% of the left-right wing shape variation could be explained by effects of pollution, independent of genetical factors.

FA of body parts point towards instabilities during development caused by various sorts of stressors (Foekema et al., 2012). The origin of variations may lie in biological and methodological factors. In this study, methodological influences causing FA, like human-induced bias, can fully be excluded as the results of the self-test displayed. By implication, the apparent variation of right-left wing asymmetry in the bumblebee samples could be pinpointed to biological origin. One of the biological origins towards the formation of FA might be attributed to genetics (Leamy & Klingenberg, 2005), like inbreeding manifestation. However, inbreeding levels were found to be very low in the present study. Even though some loci showed lower H_o than H_e, the overall heterozygosity remained high. This observation was also supported by the inbreeding coefficient F_{IS} oscillating around null. In any case, considerable inbreeding or outbreeding effects can be excluded. Prior to evaluation, we excluded any misleading markers and made sure that the number of properly scored loci were evenly distributed across populations. The good quality of the markers used in this study was further assured by the results of the HWE and LD tests; virtually no evolutive force operated on the loci selected. Synoptically, it is unlikely that the bumblebee's genetics (here: MLH) impacted the FA correlation results.

Based on the data of this study regarding the individual level, the selected POPs & mercury only had an impact on the forewing shape, thus enhancing the left-right imbalance. Struck et al. (in prep.) found a similar result for *B. lucorum* and *B. cryptarum*, even though the sampling numbers were not representative. Other studies illustrated negative correlations between heavy metal pollution and wing size variability differences (Novicic et al., 2012). It hence could be expected that different pollutants and certain combinations thereof to act differently, either boosting the left-right wing shape, the size asymmetry or both. The effects observed exclusively in the forewings can be explained by examining the developmental modules: Fore- and hindwings of bees develop from

separate imaginal disks (Klingenberg et al., 2001), leading to distinct susceptibility during development.

The relationship between FA and pollution is rooted in a geographical effect. Szentgyorgyi et al. showed (2011) that the amount of heavy metal in the bodies of bumblebees is positively correlated with the heavy metal pollution in that specific area. This is comparable with the data of the present study: The relative strength and direction of the correlation between FA and pollutants can be attributed to the POP & mercury concentrations measured within the bumblebee body pools and consequently to the sampling site.

Differences on species level. Pollution burden and the implications for the fitness proxy (here: FA index) in bumblebees revealed species-specific differences. At the Hoher Sonnblick, *B. wurflenii* bears an almost five-time higher load of BDE209 than *B. pratorum*, whose burden is at the quantification limit. With few exceptions, *B. wurflenii* in general showed higher pollution values and more pronounced individual FA values at the more strongly contaminated sampling site, Hoher Sonnblick, compared to *B. pratorum*. The reasons for this incidence might be ascribed to biological factors. One difference between both species is their nesting manner. While *B. wurflenii* mostly nests underground, the probability to get in physical contact with soil-bound pollutants is much higher. This comparison showcases the relevance of analyzing pollution effects at the species level. Pooling both bumblebee species (*Bombus* spp.) and performing one chemical analysis instead of several would require less biomass and the sampling procedure would thus be less invasive. However, without examining the bumblebee samples on species level, the establishment of species-adjusted conservation is impossible.

Conclusions & open questions. This study emphasized the importance of species-specific chemical analyses to relate pollution levels to fitness proxies. In order to be able to reveal such processes even more precisely in the future, an advancement in chemical analysis methods towards higher resolution is required, since the sampling effort would require less biomass. As an important side effect, population pools could be further split, with the consequence that chemical values could be determined on individual level. This again helps to establish direct links to individual fluctuating asymmetries. The inclusion of other fitness proxies would complement the big picture. In the design of this field-study, only correlations were revealed, no cause-and-effect relationships. For the latter purpose, we suggest the conduction of laboratory studies with the same POP & mercury ambient concentrations as in the habitats of the insects and to include parameter gradients (e.g., temperature, food supply, humidity) to record sublethal inferences within bumblebee populations over generations. For now, the residual 80% of explanation for phenotypic instabilities in the bumblebee wings remain unexplored. We assume other environmental stressors operating, such as climate

change or food shortage, both known to expose bumblebee species to detrimental stress (Rotheray et al., 2017; Soroye et al., 2020). However, 20% of variation explained for phenotypic instabilities by pollution is nevertheless considerable and can have a strong impact on natural populations, especially in the longer term.

Mountain regions are currently experiencing some of the most drastic impacts of climate change (Beniston, 2003). With atmospherical pollution joining in, specialized mountainous species, like *B. wurflenii*, are particularly imperiled. Unlike ubiquitous species, e.g., *B. pratorum*, they have less chance to circumvent contaminated and unfavorable habitats. As climate change, POP distribution and other threats do not stop at country borders, we will be invoked to address approaches on a cross-national and cross-continental scale to ensure the longer-term survival of specialized species.

Box 1: High species-composition turnover & bumblebee scarcity syndrome

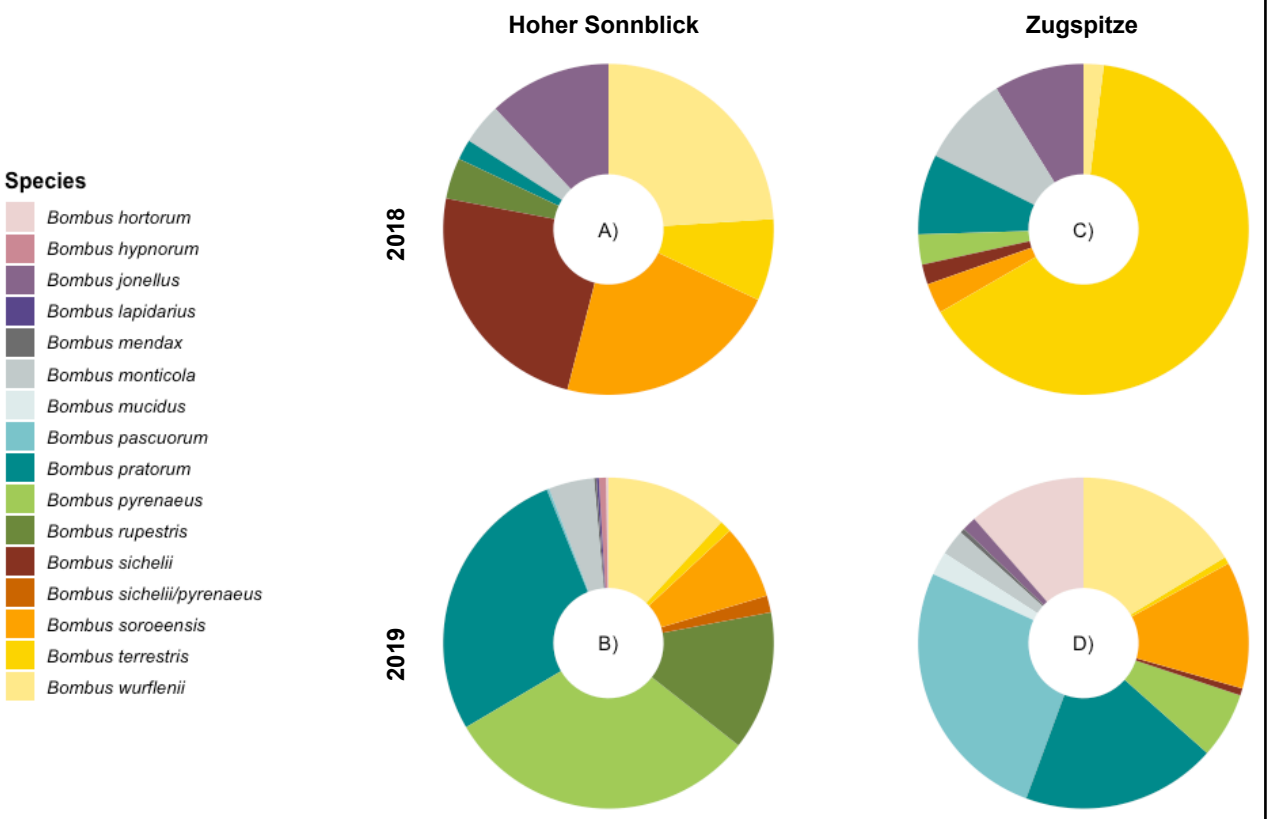

Fig. 1: *Species composition of worker bumblebees at Hoher Sonnblick (Austria) and Zugspitze (Germany) over two years. «Bombus terrestris» represents the B. terrestris-complex (B. lucorum, B. terrestris, B. cryptarum and B. magnus). «Bombus sichelii/pyrenaeus» contains individuals of B. pyrenaeus or B. sichelii that couldn't certainly attributed to either of them.* ***A)*** *Eight different species were caught in 2018 at Hoher Sonnblick with a total sample size of n = 50.* ***B)*** *Thirteen species were found at Hoher Sonnblick in 2019, n= 427.* ***C)*** *In the year 2018, eight species were recorded at Zugspitze with a sample size of 102,* ***D)*** *and twelve the year later (2019), with a sample size of 435.*

The pilot study by Struck et al. (in prep.) in the year 2018 aimed at establishing a methodological framework and additionally served to identify the species composition at the two sampling sites. The *Bombus terrestris*-complex was the most abundant species complex over both sampling sites in 2018 (Fig. 1 A & C). Based on these results, the same species were targeted in the subsequent year. To our surprise, the *B. terrestris*-complex represented only a minority of all species caught in 2019 (Fig. 1 B & D). The impact of the previous year can be excluded, due to a prolonged sampling period to keep the pressure on the nests low. However, the sampling time itself differed. In the year 2019, the bumblebees were collected at the expected peak of population size and strength in July and August, whereas in 2018, the sampling was carried out in September and, therefore, late in the season, when population size has already been degrading (Struck et al., in prep.). This sampling-date effect can also be excluded though, because both early (e.g., *B. terrestris*) as well as late (e.g., *B. sichelii*) appearing species were present in comparatively high numbers in 2018 that obviously vanished in the year 2019. Pollinators living in high elevation ecosystems seem therefore to underly a high, yearly species composition turnover.

Many high-elevated field patches, where lots of pollinators (including bumblebees) flew in 2018, were

abandoned or only sparsely inhabited by bumblebees in 2019, despite a high floral availability. Rasmont & Iserbyt (2012) have made similar observations for over a decade, calling it the bumblebee scarcity syndrome — the local extinction of whole bumblebee communities. One reason for this incidence might be the late snowfall in spring 2019. During the solitary phase, bumblebees are particularly vulnerable. Bumblebee queens of early species are rather predestinated to be caught off-guard by weather change. Low intake of food, while a lot of energy for thermoregulation would be needed, is really harmful for the queens' health. Consequently, no colony can be built up due to death from starvation. Food shortage is actually hypothesized to be one of the main causes of bumblebee population depletion (Williams et al., 2009). Iserbyt & Rasmot (2012) showed clear correlations between climatic factors and bumblebee occurrences. Bumblebees are cold-adapted bees that suffer from extreme weather situations contingent on climate change. Hence, another potential explanation might be heat waves during summer months 2019 and in consequence, a limit in water supply.

These observations have implications for the design and conduction of field experiments with bumblebees. Our data present only a snapshot, and it would be more exploratory to monitor the species composition of Hoher Sonnblick and Zugspitze over years to draw more broadly based conclusions or, at least, to recognize tendencies.

Box 2: Additional evidence for environmental stress

The present study showed that barely 20% of the left-right wing shape variation could be explained by combined effects of pollution and genetics, albeit genetic impacts could largely be excluded. For the residual 80% we assume other environmental pressures operating, such as climatic turbulences or competition for food sources that affect bumblebee species to different extent.

Another hint that pleads for additional environmental stressors in the analyzed populations provides the weight listing (Table 1): Bumblebee weights weren't consistently proportional to the number of worker specimens collected. For example, a worker bee from *Bombus wurflenii* weighed almost twice as much at the Hoher Sonnblick compared with the Zugspitze. The same effect was found for *B. pratorum*, but in the opposite direction relating to the sampling sites.

Table 1: *Collected pool samples expressed in numbers: weight and number of individuals caught, grouped by species and sampling location.*

species	sampling location	# individuals	pool weight (g)	Ø weight (g) per individual
Bombus wurflenii	Zugspitze	71	7.896	0.1112
	Hoher Sonnblick	51	10.162	0.1993
Bombus pratorum	Zugspitze	83	11.845	0.1427
	Hoher Sonnblick	126	8.521	0.0676

Body mass in bumblebees correlates with body size. Since body size is a key trait across bee taxa, it is strongly connected to fitness parameters. Smaller body sizes influence, amongst others, the foraging ability, the mating and the hibernation success (Pyke, 1978; Müller & Schmid-Hempel, 1992; Baer et al., 2003; Cueva del Castillo et al., 2015). On one hand, size declines can be caused by a broad spectrum of environmental factors, such as scarce resource availability, extreme temperature amplitudes and pathogen burden (Sutcliffe & Plowright, 1988; Cueva del Castillo et al., 2015; Chole et al., 2019). Landscape fragmentation, on the other hand, favors usually the production of larger bumblebees (Greenleaf et al., 2007; Persson & Smith, 2011; Gérard et al., 2021). Generally speaking, variations in body size can be an early indicator of environmental stress (Grab et al., 2019).

Changes in body size also occur naturally throughout the flowering season in a bumblebee colony. After a successful colony founding, a young queen faces the size-number trade-off in reproduction: The first hatching worker bees are relatively small and initially display the role of all-rounders. As soon as the colony gets stronger, worker bees split up in tasks. The specimens that fly out to forage have distinguishably larger body sizes — except for offsprings of weak colonies. In our case, seasonal effects could be eliminated, because the sampling periods of the two regions actually overlapped.

These observations about variations in body mass (Table 1) alone could still suggest local adaptions. However, the FA results of fore- and hindwing sizes (Table 2) consistently support the trend displayed in weight. Like body size, fluctuating asymmetry in wings also stands for instabilities caused by all kinds of stressors (Klingenberg, 2003; Beasley et al., 2013). This link gets clearer when looking at the example *B. wurflenii*: The variation that explained left-right wing size differences in individuals caught at the Zugspitze was almost 25 and 6 times (for forewings and hindwings, respectively) higher than variation explained by individuals (printed in bold type, Table 2). In contrast, however, the variation in wing sizes at Hoher Sonnblick was higher among individuals, which is actually expectable under normal circumstances. Thus, the *B. wurflenii* population at the Zugspitze had been subject to certain stressors. The same is true for *B. pratorum* at the Hoher Sonnblick. Both species obviously react with a differing sensitivity to the pressures prevailing on both mountains. This again emphasizes the importance to conduct investigations that deal with the interplay of complex ecosystem factors on species level.

Table 2: *Results of the Procrustes analyses of variance (ANOVA) of wing sizes in B. wurflenii and B. pratorum. SS, sum of squares; MS, mean sum of squares; df, degrees of freedom; F, variation; p, p-value. Populations, where fluctuating asymmetry explained more variation than other classifiers, are marked with a star (*) and the relevant F-values printed in bold.*

	wing	location	classifier	SS	MS	df	F	p
Bombus wurflenii	forewings	Zugspitze *	Individual	377169.5513	5802.6085	65	27.56	< 0.0001
			Fluctuating asymmetry	13683.1129	210.5094	65	675.84	< 0.0001
			Imaging error	38.0001	0.3115	122		
		Hoher Sonnblick	Individual	182101.4159	4138.6685	44	123.66	< 0.0001
			Fluctuating asymmetry	1472.6094	33.4684	44	115.56	< 0.0001
			Imaging error	24.0375	0.2896	83		
	hindwings	Zugspitze *	Individual	51828.4045	762.1824	68	25.05	< 0.0001
			Fluctuating asymmetry	2069.1469	30.4286	68	167.99	< 0.0001
			Imaging error	23.3657	0.1811	129		
		Hoher Sonnblick	Individual	20973.8286	466.0851	45	64.94	< 0.0001
			Fluctuating asymmetry	322.9691	7.1771	45	37.46	< 0.0001
			Imaging error	16.8595	0.1916	88		
Bombus pratorum	forewings	Zugspitze	Individual	598130.1525	8927.3157	67	212.59	< 0.0001
			Fluctuating asymmetry	2813.5670	41.9935	67	148.63	< 0.0001
			Imaging error	32.7745	0.2825	116		
		Hoher Sonnblick *	Individual	647972.2823	6479.7228	100	29.05	< 0.0001
			Fluctuating asymmetry	22307.7297	223.0773	100	261.32	< 0.0001
			Imaging error	153.6606	0.8537	180		
	hindwings	Zugspitze	Individual	72989.5986	973.1946	75	74.46	< 0.0001
			Fluctuating asymmetry	980.2307	13.0697	75	61.46	< 0.0001
			Imaging error	28.2820	0.2126	133		
		Hoher Sonnblick *	Individual	72598.9940	698.0673	104	15.78	< 0.0001
			Fluctuating asymmetry	4601.1581	44.2419	104	202.70	< 0.0001
			Imaging error	42.1237	0.2183	193		

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Appendix

LAND SALZBURG

Frau
Veronika Hierlmeier, MSc.
Bayerisches Landesamt für Umwelt
Bayerisches Landesamt für Umwelt
Dienststelle Garmisch-Partenkirchen
Gsteigstraße 43
82467 GARMISCH-PARTENKIRCHEN
DEUTSCHLAND

Nationalparkverwaltung
Hohe Tauern

Zahl (Bitte im Antwortschreiben anführen)
20507-96/47/9-2019

Datum
08.08.2019

Betreff
Bescheid - Änderung Arten
Probennahme verschiedener Insektenarten, Projekt "protectAlps"

Gerlos Straße 18
5730 Mittersill
Fax +43 6562 40849-40
nationalpark@salzburg.gv.at
Mag. Claudia Kranawendter, LLB.oec
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BESCHEID

I. Spruch

Auf Antrag von Frau Veronika Hierlmeier, Msc., Bayerisches Landesamt für Umwelt vom 31.07.2019 wird von der Salzburger Landesregierung der Bescheid vom 04.06.2019, Zl. 20507-96/47/7-2019 mit dem Frau Veronika Hierlmeier, Msc., Bayerisches Landesamt für Umwelt die nationalparkrechtliche Ausnahmebewilligung für die Durchführung einer selektiven Probennahme verschiedener Insektenarten zur Untersuchung des Einflusses von chemischen Stressoren auf Insekten im Rahmen des INTER-REG-A-Projektes „protectAlps“ in der Nationalpark-Außenzone im Bereich Kolm Saigurn des hinteren Hüttwinkltales, Gemeinde Rauris, unter Vorschreibung von Auflagen und Befristungen erteilt wurde, im Spruchabschnitt II Auflagen und Befristungen, im Auflagenpunkt 3 **wie folgt abgeändert**:

3. Die Bewilligung für das Aufsammeln und die Entnahme der Insekten ist auf die im gegenständlichen Antrag angeführten Methoden, Arten bzw. Artengruppen und Mengen auf das unbedingt notwendige Minimum beschränkt. Zusätzlich den angesuchten Arten bzw. Artengruppe darf eine Beprobung der Gattung Bombus spp. erfolgen. Eine über die Fangmethoden hinausgehende Verletzung von Tieren ist unbedingt zu vermeiden.

Appendix 1. *Collective permission from Nationalpark Hohe Tauern, version 1 (04.06.2019).*

LAND SALZBURG

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Zahl (Bitte im Antwortschreiben anführen)
20507-96/47/7-2019

Datum
04.06.2019

Betreff
Bescheid
Probennahme verschiedener Insektenarten, Projekt "protectAlps"

BESCHEID

Spruch

I. Auf Antrag vom 30.07.2018, sowie Ergänzungen vom 06.08.2018 und 20.08.2018 eingebracht durch Veronika Hierlmeier, Bayerisches Landesamt für Umwelt, Gsteigerstraße 43, 82467 Garmisch-Partenkirchen, Deutschland, wird von der Salzburger Landesregierung die

nationalparkrechtliche Ausnahmebewilligung

zur Durchführung einer selektiven Probennahme verschiedener Insektenarten zur Untersuchung des Einflusses von chemischen Stressoren auf Insekten im Rahmen des INTERREG-A-Projektes „protectAlps“ in der Nationalpark-Außenzone im Bereich Kolm Saigurn des hinteren Hüttwinkltales, Gemeinde Rauris, nach Maßgabe des Antrags, sofern die nachfolgenden Auflagen und Bedingungen nichts Anderes bestimmen, erteilt:

II. Auflagen und Befristungen:

1. Die Bewilligung gilt ausschließlich für die im Ansuchen angeführte Projektgruppe im Rahmen des INTERREG-A-Projektes „protectAlps“ unter der Leitung von Antragstellerin Frau Veroni-

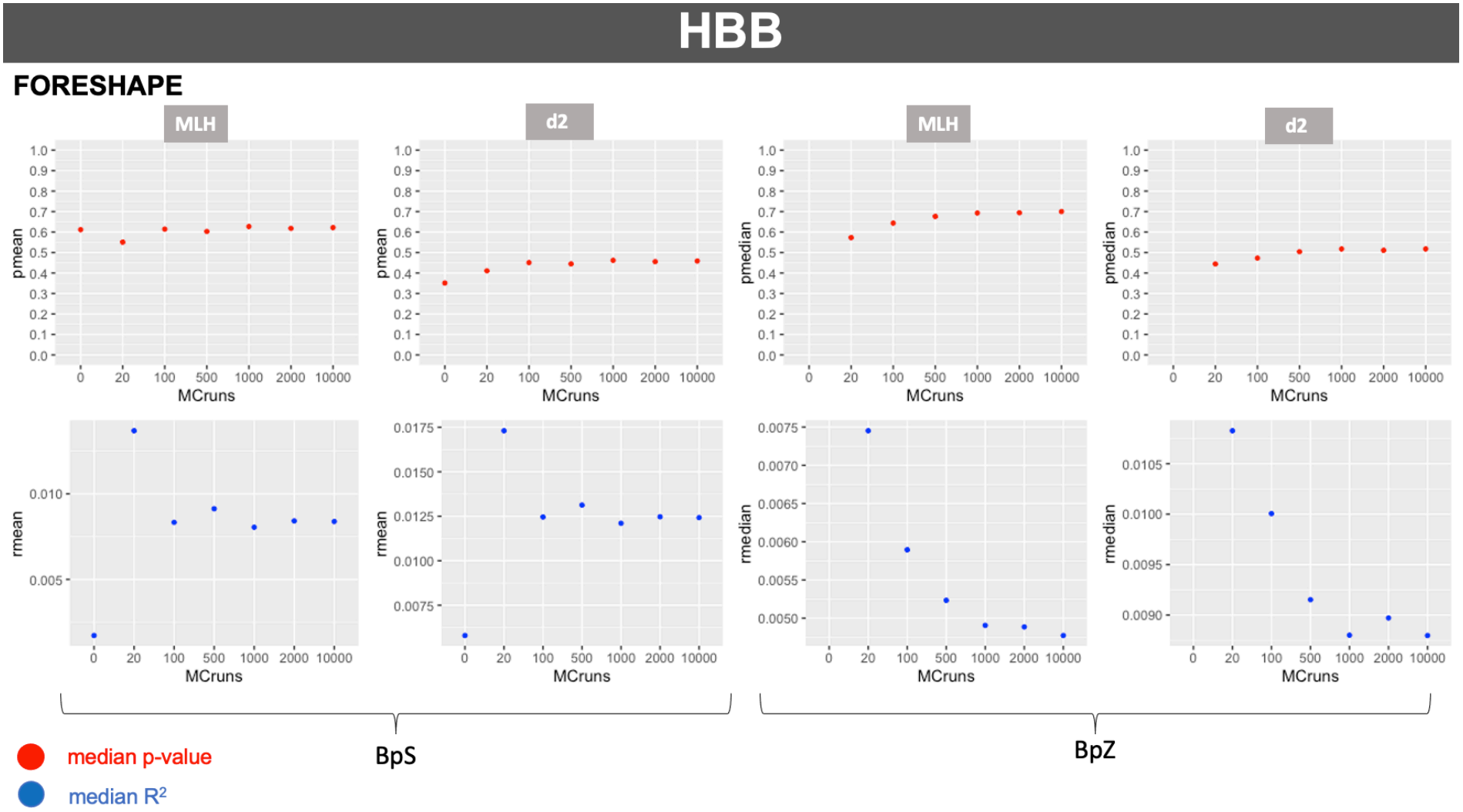
Appendix 2. *Collective permission from Nationalpark Hohe Tauern, version 2 (08.08.2019).*

Appendix 3. *Persistent organic pollutants (POPs) studied, their abbreviations and the chemical group they belong to. The pollutants shaded in gray could not be detected in the bumblebee samples in this study.*

Chemical Name (Abbreviation)	Chemical Group
p-TBX	Brominated flame retardants (BFRs)
PBT	Brominated flame retardants (BFRs)
PBEB	Brominated flame retardants (BFRs)
HBB	Brominated flame retardants (BFRs)
PBB 153	Brominated flame retardants (BFRs)
syn-DP	Brominated flame retardants (BFRs)
anti-DP	Brominated flame retardants (BFRs)
DBDPE	Brominated flame retardants (BFRs)
BDE-28	Brominated flame retardants (BFRs)
BDE-47	Brominated flame retardants (BFRs)
BDE-49	Brominated flame retardants (BFRs)
BDE-66	Brominated flame retardants (BFRs)
BDE-77	Brominated flame retardants (BFRs)
BDE-85	Brominated flame retardants (BFRs)
BDE-99	Brominated flame retardants (BFRs)
BDE-100	Brominated flame retardants (BFRs)
BDE 118	Brominated flame retardants (BFRs)
BDE 126	Brominated flame retardants (BFRs)
BDE 139	Brominated flame retardants (BFRs)
BDE 153	Brominated flame retardants (BFRs)
BDE 154	Brominated flame retardants (BFRs)
BDE 181	Brominated flame retardants (BFRs)
BDE 183	Brominated flame retardants (BFRs)
BDE 196	Brominated flame retardants (BFRs)
BDE 197	Brominated flame retardants (BFRs)
BDE 203	Brominated flame retardants (BFRs)
BDE 207	Brominated flame retardants (BFRs)
BDE 209	Brominated flame retardants (BFRs)

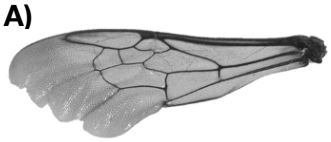
2,4,4´-Trichlorbiphenyl (PCB 28)	Polychlorinated biphenyls (PCBs)
2,2´,5,5´-Tetrachlorbiphenyl (PCB 53)	Polychlorinated biphenyls (PCBs)
2,2´,4,5,5´-Pentachlorbiphenyl (PCB 101)	Polychlorinated biphenyls (PCBs)
2,2´,3,4,4´,5´-Hexachlorbiphenyl (PCB 138)	Polychlorinated biphenyls (PCBs)
2,2´,4,4´,5,5´-Hexachlorbiphenyl (PCB 153)	Polychlorinated biphenyls (PCBs)
2,2´,3,4,4´,5,5´-Heptachlorbiphenyl (PCB 180)	Polychlorinated biphenyls (PCBs)
Aldrin	Organochlorine pesticides (OCPs)
Dieldrin	Organochlorine pesticides (OCPs)
Endrin	Organochlorine pesticides (OCPs)
alpha-HCH	Organochlorine pesticides (OCPs)
beta-HCH	Organochlorine pesticides (OCPs)
gamma-HCH (Lindan)	Organochlorine pesticides (OCPs)
delta-HCH	Organochlorine pesticides (OCPs)
epsilon-HCH	Organochlorine pesticides (OCPs)
alpha-Endosulfan	Organochlorine pesticides (OCPs)
beta-Endosulfan	Organochlorine pesticides (OCPs)
Endosulfan-sulfat	Organochlorine pesticides (OCPs)
Hexachlorbutadien	Organochlorine pesticides (OCPs)
Pentachlorbenzol	Organochlorine pesticides (OCPs)
Hexachlorbenzol	Organochlorine pesticides (OCPs)
Octachlorstyrol	Organochlorine pesticides (OCPs)
Mirex	Organochlorine pesticides (OCPs)
o,p´-DDD	Organochlorine pesticides (OCPs)
o,p´-DDE	Organochlorine pesticides (OCPs)
o,p´-DDT	Organochlorine pesticides (OCPs)
p,p´-DDD	Organochlorine pesticides (OCPs)
p,p´-DDE	Organochlorine pesticides (OCPs)
p,p´-DDT	Organochlorine pesticides (OCPs)
Lambda-Cyhalothrin	Organochlorine pesticides (OCPs)
o,p´-Methoxychlor	Organochlorine pesticides (OCPs)
p,p´-Methoxychlor	Organochlorine pesticides (OCPs)
Pentachloranisol	Organochlorine pesticides (OCPs)

cis-Chlordan	Organochlorine pesticides (OCPs)
trans-Chlordan	Organochlorine pesticides (OCPs)
oxy-Chlordan	Organochlorine pesticides (OCPs)
Heptachlor	Organochlorine pesticides (OCPs)
cis-Heptachlorepoxid	Organochlorine pesticides (OCPs)
trans-Heptachlorepoxid	Organochlorine pesticides (OCPs)
PF6C	Perfluorochemicals (PFCs)
PF7C	Perfluorochemicals (PFCs)
PF8C	Perfluorochemicals (PFCs)
PF9C	Perfluorochemicals (PFCs)
PF10C	Perfluorochemicals (PFCs)
PF11C	Perfluorochemicals (PFCs)
PF12C	Perfluorochemicals (PFCs)
PF4S	Perfluorochemicals (PFCs)
PF6S	Perfluorochemicals (PFCs)
PF8S	Perfluorochemicals (PFCs)
PF5S	Perfluorochemicals (PFCs)



Appendix 4. *An example of different model outputs for the respective Monte Carlo runs. The following variables are integrated in the model outputs shown: the FA values of the forewing shape of Bombus pratorum individuals, either from the Sonnblick (BpS), or the Zugspitze population (BpZ); both inbreeding coefficient (MLH and d2); and the brominated flame retardant HBB as potential environmental stressor. Red dots: Median of the p-values. Blue dots: Median R^2-values. MCruns: Number of Monte Carlo simulation runs.*

A)



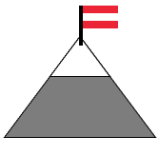
Classifiers used for the Procrustes ANOVA:
Individuals: individual
Sides: side
Error 1: updown

Centroid size:

Effect	SS	MS	df	F	P (param.)
Individual	440210.797530	24456.155418	18	111.30	<.0001
Side	555.959271	555.959271	1	2.53	0.1291
Ind * Side	3955.054088	219.725227	18	660.88	<.0001
Error 1	12.301435	0.332471	37	0.56	0.9718
Residual	42.101512	0.592979	71		

Shape, Procrustes ANOVA:

Effect	SS	MS	df	F	P (param.)
Individual	0.04629408	0.0000714415	648	5.88	<.0001
Side	0.00047168	0.0000131021	36	1.08	0.3491
Ind * Side	0.00787116	0.0000121469	648	12.97	<.0001
Error 1	0.00124714	0.0000009363	1332	1.24	<.0001
Residual	0.00192284	0.0000007523	2556		



Classifiers used for the Procrustes ANOVA:
Individuals: individual
Sides: side
Error 1: updown

Centroid size:

Effect	SS	MS	df	F	P (param.)
Individual	116366.596305	6845.093900	17	2.32	0.0458
Side	5527.175602	5527.175602	1	1.87	0.1888
Ind * Side	50132.969905	2948.998230	17	4002.55	<.0001
Error 1	25.050503	0.736780	34	0.01	1.0000
Residual	5548.435283	88.070401	63		

Shape, Procrustes ANOVA:

Effect	SS	MS	df	F	P (param.)
Individual	0.04989696	0.0000815310	612	2.43	<.0001
Side	0.00139924	0.0000388677	36	1.16	0.2456
Ind * Side	0.02054318	0.0000335673	612	1.70	<.0001
Error 1	0.02420740	0.0000197773	1224	0.94	0.8753
Residual	0.04754311	0.0000209626	2268		

B)

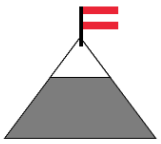
Classifiers used for the Procrustes ANOVA:
Individuals: individual
Sides: side
Error 1: updown

Centroid size:

Effect	SS	MS	df	F	P (param.)
Individual	49933.332727	2628.070144	19	35.93	<.0001
Side	5.675111	5.675111	1	0.08	0.7836
Ind * Side	1389.856747	73.150355	19	305.07	<.0001
Error 1	9.591254	0.239781	40	0.78	0.7988
Residual	23.843858	0.305690	78		

Shape, Procrustes ANOVA:

Effect	SS	MS	df	F	P (param.)
Individual	0.08593639	0.0005653710	152	8.13	<.0001
Side	0.00062162	0.0000777024	8	1.12	0.3543
Ind * Side	0.01056586	0.0000695122	152	6.86	<.0001
Error 1	0.00324028	0.0000101259	320	1.56	<.0001
Residual	0.00405575	0.0000064996	624		



Classifiers used for the Procrustes ANOVA:
Individuals: individual
Sides: side
Error 1: updown

Centroid size:

Effect	SS	MS	df	F	P (param.)
Individual	32191.230965	1694.275314	19	4.60	0.0008
Side	582.861904	582.861904	1	1.58	0.2237
Ind * Side	6998.463299	368.340174	19	1147.16	<.0001
Error 1	12.843557	0.321089	40	0.05	1.0000
Residual	557.220742	6.965259	80		

Shape, Procrustes ANOVA:

Effect	SS	MS	df	F	P (param.)
Individual	0.05615079	0.0003694131	152	3.43	<.0001
Side	0.00111054	0.0001388170	8	1.29	0.2527
Ind * Side	0.01635771	0.0001076165	152	14.22	<.0001
Error 1	0.00242183	0.0000075682	320	1.07	0.2451
Residual	0.00453631	0.0000070880	640		

Appendix 5. *Procrustes ANOVA outputs of the pilot data set of a total of 40 individuals of Bombus pratorum, half split between the two sampling sites. Values concerning the digitizing (shown as „Residual“) and the imaging error (indicated as „Error 1“) are outlined in red.* ***A)*** *Results of the forewing landmarking; left, the results from the Zugspitze population, right, those from the Hoher Sonnblick population.* ***B)*** *Results of the hindwing landmarking; left, the results from the Zugspitze population, right, those from the Hoher Sonnblick population. Abbreviations: SS, Sum of Squares; MS, Mean Sum of Squares; df, degrees of freedom; F, variation; P (param.), p-value.*