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„Genetic and spatial analyses of two population patches in the fire salamander (*Salamandra salamandra*) from the Vienna woods (Austria): Groupwise vs. individual based approach“

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Judith Bürgler, BSc

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Contents

Genetic and spatial analyses of two population patches in the fire salamander (*Salamandra salamandra*) from the Vienna woods (Austria): Groupwise vs. individual based approach

Judith Bürgler, BSc

University of Vienna

Department of Integrative Zoology

Althanstraße 14, A-1090 Vienna, AUSTRIA

Abstract

Most animal populations in the wild show a patchy distribution. As spatial data alone do not allow drawing conclusions about actual patterns of gene flow, molecular methods have become of significant importance when studying animal populations. In this study we investigated a fire salamander population from the Vienna Woods that appeared spatially clustered. The respective regions where the individuals were found differed in their possibilities for larval deposition: small flowing streams ("Stream") in the West and temporary ponds ("Pond") in the East. We examined if this spatial clustering actually resembles the presence of separate reproductive groups, by using spatial locations and microsatellite genotypes of fire salamander individuals from both patches. We used common population genetic measures, such as F_{ST} and F_{IS}, and additionally an individual based approach to investigate patterns of possible genetic differentiation between the two population patches. Therefore, we determined pairwise spatial distances and pairwise relatedness values between all possible pairs of salamanders. While F_{ST} failed to detect genetic differentiation between the patches, the individual based approach

revealed that pairwise relatedness was strongly negatively correlated with spatial distance. Pair relatedness was significantly higher within, than between the groups. This effect was stronger for the “Pond” than for the “Stream” individuals. Our results show that reproductive groups and population subdivision among the two population patches may exist, despite little overall genetic differentiation.

Keywords: Salamandra salamandra, reproductive groups, amphibians, genetic differentiation

Introduction

Population subdivision is often caused by environmental patchiness, such as areas of favorable habitat intermixed with unfavorable habitats, but can also be caused by social behaviour (Hartl & Clark, 2007). When population subdivision occurs due to geographic isolation, this might lead to genetic differentiation between the subpopulations due to the loss of gene flow between such patches and genetic drift within each subpopulation, so that the allele frequencies evolve independently (Hartl & Clark, 2007; Hamilton, 2009). Genetic differentiation can furthermore lead to sympatric speciation. Mayr (1963) defined sympatric speciation as the evolution of reproductive isolation without geographic isolation. It is one of the most fundamental, albeit poorly understood topics in evolutionary biology. It occurs when behaviour, due to ecological constraints, mediates population subdivision by limiting the gene flow between subpopulations. The main elements that influence genetic population structure are mating, genetic drift and gene flow (Templeton, 2006). These three processes predict the effects of gene flow by determining the extent to which populations will genetically diverge or converge (Freeland et *al*, 2011). Subpopulations are not genetically isolated from

one another if there is migration of individuals among the subpopulations, resulting in gene flow between patches. In many species the amount of gene flow between populations is inversely proportional to the geographic distances between them as individuals are most likely to spread to nearby sites (Freeland et *al*, 2011). This is known as isolation by distance (IBD; Wright, 1943). Mobility of individuals is another factor that affects gene flow between populations.

The subject of our study, the fire salamander (*Salamandra salamandra),* is present across most of central, western and southern Europe. In Austria, the fire salamander can be found in altitudes between 200 and 700 m (Cabela et al. 2001). Currently it is protected over most of its range by national legislations, which is the case in Austria, although on an international scale it is listed as "Least Concern" by the IUCN Red List (IUCN 2011).The threats concerning its distribution are all of anthropogenic nature. They include the pollution of breeding sites, general habitat destruction and population fragmentation. The aspect of habitat fragmentation is of great importance, as it can lead to genetic consequences due to bottleneck effects. For instance in the critically endangered frog *Rana sevosa* a case of significantly lower genetic variation after habitat fragmentation has been described (Richter et al., 2009). *Salamandra salamandra* is dependent on old broadleaf forests that supply both terrestrial environment to provide shelter, and aquatic larval deposition sites. Small streams serve as main larval habitats until metamorphosis is completed (Cabela et al. 2001). In contrast, all following adult stages are entirely terrestrial and therefore not attached to the aquatic habitat (Thiesmeier, 2004). It is therefore essential to protect not only the breeding sites of *S. salamandra*, but also the surrounding habitats, and in general to control human impact on their migratory routes. Mating occurs in spring to early summer and eggs are internally fertilized in the mother's oviduct. Next, the

zygotes develop into complete larvae within the mother, and will be deposited into suitable aquatic habitats in spring of the following year (Steinfartz et al. 2007). Interestingly, Steinfartz et al. (2007) detected and studied a population of fire salamanders, in which also pond reproduction has evolved as a habitat-specific adaptation following the last glaciation (Weitere et al. 2004). They found genetic differentiation between pond- and stream- reproducing salamanders within the same forest population and thus hypothesized that sympatric speciation might occur. In Austria we found a similar setup: a small forest patch that offered ponds and streams for larval deposition. Our study area is located in Liesing, the 23rd district of Vienna, and consists of two different aquatic sites for larval deposition, that offer two distinct reproductive niches, stream and pond breeding. There are no spatial structures to inhibit the movement of adult fire salamander between those two localities. Therefore, we hypothesized that spatial clustering actually resembles the presence of two separate reproductive groups, subsequently resulting in genetic differentiation between the two investigated population patches.

Material and methods

Study sites

Our study area is located in Liesing, at the south–west edge of Vienna (+48°9 ´6.58", +16°14´46.62"), of approximately 1, 5 km², characterised by oak and hornbeam forest. Water bodies are available as small streams and temporary ponds. To the north the study area is constrained by the wall of the wildlife park "Lainzer Tiergarten", to the south and west it is defined by large meadows, and to the east side by urban area. Samples were taken from two locations, the stream site (Stream) in the west and the pond site (Pond) in the east of our study area.

There were no major spatial barriers that could have hampered migration of adult salamanders between both sites (Figure 1).

Sampling

Sampling took place between March 2010 and June 2011. At the two sites, we sampled in total 546 individuals. With cotton swabs saliva of each individual was collected. The swabs were then deposited in small tubes and stored at -20°C until DNA extraction. Individuals were sexed by phenotypic distinction marks such as swollen cloacae (males) or thick bellies (gravid females). Individuals that were too small to be unambiguously sexed were recorded as juveniles.

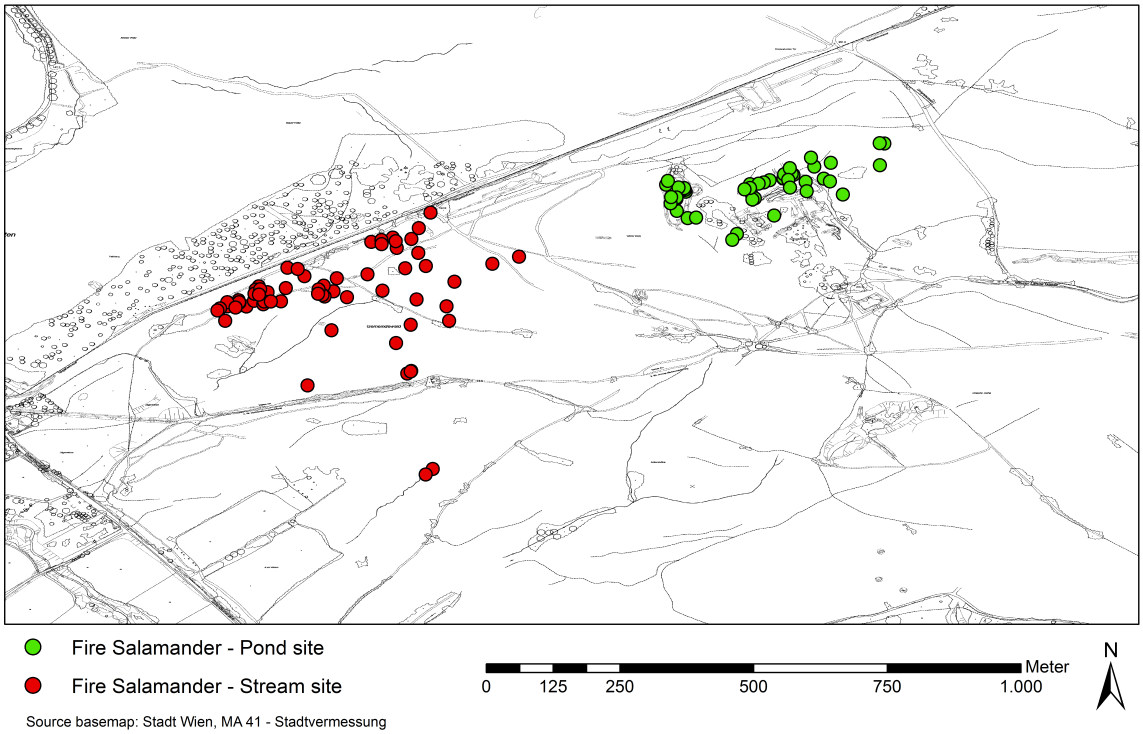

Figure 1: Map of study area at Liesing, Vienna, with recordings of sampled individuals at the stream site and pond site locations.

Genotyping

Genomic DNA was isolated from all cotton swabs by using a Proteinase K digestion followed by a standard phenol-chloroform protocol. For the PCR amplifications we diluted the samples to a final concentration of 10-50 ng/µl. Microsatellite genotypes of nine loci (*Sal E2, Sal E6, Sal E7, Sal E8, Sal E12, Sal E14, Sal 3, Sal 23, Sal 29*) were obtained using PCR primers and slightly adapted protocols as described in Steinfartz *et al.* (2004) (Table 1). Each 10µl amplification reaction contained: 1µl genomic DNA, 1µl of each primer, 1µl dNTPs (2 mM each), 0.05 µl Taq (0, 1 U), 0.6 µl $MgCl_2$, 1 µl 10 x RB buffer and 4.35 µl dH_2O. We ran the following PCR program: 94 °C for 4 min, 39 cycles of 94 °C for 45 sec, AT °C for 45 sec, and 72 °C for 45 sec. After the final cycle an additional extension step of 72 °C for 5 min was added. The locus specific annealing temperatures (AT) for each primer set are shown in table 1. The amplified products were diluted with water, mixed with internal size standard ROX500, run on an ABI 3130xl sequencer and analysed using Peak Scanner 1.0 (Applied Biosystems). All loci were visually identified, ambiguous alleles were re-genotyped and the final allele sizes were determined using the binning software Tandem 1.01 (Matschiner & Salzburger 2009). Only genotypes with at least 6 amplified loci were used.

Table 1: List of the nine microsatellite loci for S*alamandra salamandra* used in this study

Locus	Primer sequences (5´-3´), fluorescence labelling	Annealing temperature
Sal E2 AY612894	F: FAM-CACGACAAAATACAGAGAGTGGATA R: ATATTTGAAATTGCCCATTTGGTA	53 °C
Sal E6 AY612885	F: HEX-GGACTCATGGTCACCCAGAGGTTCT R: ATGGATTGTGTCGAAATAAGGTATC	58 °C
Sal E7 AY612886	F: HEX-TTTCAGCACCAAGATACCTCTTTTG R: CTCCCTCCATATCAAGGTCACAGAC	53 °C
Sal E8 AY612887	F: NED-GCAAAGTCCATGCTTTCCCTTTCTC R: GACATACCAAAGACTCCAGAATGGG	58 °C
Sal E12 AY612889	F: NED-CTCAGGAACAGTGTGCCCCAAATAC R: CTCATAATTTAGTCTACCCTCCCAC	58 °C
Sal E14 AY612890	F: HEX-GCTGCCCTCTCTGCCTACTGACCAT R: GCCAAGACATGGAACACCCTCCCGC	65 °C
Sal 3 AY612891	F: FAM-CTCAGACAAGAAATCCTGCTTCTTC R: ATAAATCTGTCCTGTTCCTAATCAG	58 °C
Sal 23 AY612893	F: FAM-TCACTGTTTATCTTTGTTCTTTTAT R: AATTATTTGTTTGAGTCGATTTTCT	50 °C
Sal 29 AY612892	F: FAM-CTCTTTGACTGAACCAGAACCCC R: GCCTGTCGGCTCTGTGTAACC	58 °C

Statistical analysis

Observed and expected heterozygosities and the mean number of alleles per locus were obtained from CERVUS 3.0.3 (Marshall et al., 1998-2007). FSTAT 2.9.3.2 (Goudet, 2002) was used to calculate probability tests such as Hardy – Weinberg equilibrium and to calculate Wright's (1965) inbreeding coefficient (F_{IS}). The degree of differentiation between populations was evaluated with Wright's (1965) index of population subdivision (F_{ST}). We applied a simple mantel test to determine a correlation between genetic relatedness and spatial distance (Mantel, 1967) using the software zt (Bonnet & Van de Peer 2002) with 100,000 randomized permutations of the residual matrix.

Additionally, we used KINGROUP (Konovalov *et al*. 2004) to define the pairwise relatedness coefficients *r* (Queller & Goodnight 1989) for all possible pairs of fire salamanders. The lowest probability of recent coalescence is represented by a negative value of -1 and the coefficient increases continuously up to a value of +1 according to the increase of genetic similarity between two individuals (Queller & Goodnight 1989; Blouin 2003; Konovalov & Heg 2008).

Results

Between March 2010 and June 2011 we sampled in total 546 individuals in the study area. For the present study saliva samples from 94 individuals (Stream: N = 45; Pond: N = 49) were genetically analyzed. The sex ratio was 27 males and 22 females at the Pond location and 30 males, 12 females and 3 juveniles at the Stream location. These samples were genotyped at 9 microsatellite loci (Table 1), yielding 2-13 alleles (mean = 7.56) per locus for the Pond location and 3-12 alleles (mean = 8) for the Stream location (Table 2). Only the locus *Sal E2* showed significant deviation from HWE (P = 0.0022, adjusted P value after Bonferroni correction = 0.00278). No linkage disequilibrium was found for any of the loci (all values above the adjusted P value of 0.001389 after Bonferroni correction). The number of alleles per locus was not significantly different between the two sites (Wilcoxon signed rank test, N = 9, W = -0.213, P = 0.831).

Table 2: Characterisation of the nine microsatellite loci

	Stream			Pond		
Locus	k	H_{obs}	H_{exp}	k	H_{obs}	H_{exp}
8	8	0.841	0.810	7	0.891	0.764
29	9	0.625	0.656	8	0.512	0.585
14	3	0.714	0.547	7	0.587	0.560
2	8	0.676	0.786	10	0.596	0.778

	3	8	0.550	0.755	2	0.600	0.502
	7	9	0.600	0.636	10	0.605	0.542
	12	12	0.737	0.798	13	0.705	0.809
	23	10	0.692	0.649	6	0.688	0.649
	6	5	0.636	0.622	5	0.558	0.680
mean		8	0.675	0.695	7.56	0.638	0.652

Stream and Pond: k, number of alleles; H_{obs}, Heterozygosity observed; H_{exp}, Heterozygosity expected; mean;

The observed heterozygosities (H_{obs}) and the expected heterozygosities (H_{exp}) for the 9 loci ranged from 0.550 to 0.841 (Table 2), and did not significantly differ between the two sites (Wilcoxon signed rank test, H_{obs}: N = 9, W = -1.364, p = 0.173; H_{exp}: N = 9, W = -1.120, p = 0.263).

F_{IS} for both locations was low (Stream: 0.023 and Pond: 0.030) indicating no loss of heterozygosity (or low inbreeding) at both study sites, respectively. The F_{ST} value was also low (0.0392, P = 0.01), suggesting low genetic differentiation between the two sampling locations. However, when applying the simple Mantel test (Mantel 1967; r = -0.1396, P<0,001), we found a significant negative correlation between pairwise relatedness and spatial distance at our study area. Furthermore, mean pairwise relatedness was significantly higher among individuals within than between sites (Wilcoxon signed rank test: Pond: N = 49; W = -6.003, p< 0,001; Stream: N = 45; W = -3.042, p = 0.002). These analyses were separately performed for Pond and Stream.

Discussion

In this study we analysed the genetic structure of a *S. salamandra* population to investigate a possible presence of reproductive clusters, i.e. the occurrence of population subdivision and possible genetic differentiation. Previously, such

observations were made in a similar setup in a German forest habitat by Steinfartz *et al*. (2007). The ecological circumstances were comparable, as in both study areas two separate aquatic structures are available for larval deposition. Hence, also the study site in Vienna provides the possibility for sympatric speciation, as there are no spatial structures to inhibit the movement of adult fire salamander while simultaneously offering two reproductive niches – stream and pond breeding. We used spatial locations and microsatellite genotypes of fire salamander individuals from those locatilies (Stream and Pond) to determine pairwise spatial distances and pairwise relatedness values between all possible pairs of salamanders in order to investigate for genetic differentiation between those localities. Our main result shows that pairwise relatedness was strongly negatively correlated with spatial distance, as pair relatedness was significantly higher within than between the groups.

Population subdivision has been linked to environmental patchiness, but it is also influenced by social behaviour (Hartl & Clark, 2007). Given that patchiness is a common feature of most habitats (freshwater lakes have shallow and deep areas, meadows have marshy and dry areas, forests have sunny and shady areas) animal populations in the wild often show a patchy distribution. Mobility of individuals is another factor that affects gene flow between populations. Among certain subpopulations these given conditions might lead to genetic differentiation due to the loss of gene flow and genetic drift (Hartl & Clark, 2007). Therefore, molecular methods have become of significant importance to draw conclusions about actual patterns of gene flow in population genetic studies. We used microsatellites for genetic analyses, because of their high variabiltiy. This approach has already been successfully applied in previous studies (Ursprung *et al*., 2011; Steinfartz *et al*., 2007). For the statistical analyses we used common population genetic measures,

such as F_{IS} and F_{ST}. The F_{IS} values found in our study (Stream: 0.023, Pond: 0.030) were low, thus indicating low degree of inbreeding and no loss of heterozygosity. According to Wright's qualitative guidelines (1978) the F_{ST} values of 0-0.05 are generally considered to indicate little genetic differentiation, values of 0.05-0.25 indicate moderate genetic differentiation and values over 0.25 represent pronounced levels of genetic differentiation. Accordingly, our F_{ST} value of 0.0392 suggests little genetic differentiation. Nevertheless, even in the face of occasional gene flow, random genetic drift will cause differentiation in the allele frequencies of subpopulations (Hartl & Clark, 2007). Considering Wright`s guidelines our F_{ST} value is by no means insignificant, because one of the limitations of F_{ST} is that it does not capture the full range of possibilities that can be found in natural populations. Additionally we applied the Mantel test, to test for a possible correlation between genetic and geographical distance (Freeland *et al*., 2011). This is an individual based genetic approach that enables much finer resolution of the relatedness between individuals. Most subpopulations are not genetically isolated from one another owing to some migration or movement of some individuals among the subpopulations, which results in gene flow between them (Hartl & Clark, 2007). Furthermore, the resulting r value (r=-0.1396, P<0,001) from the Mantel test shows a significant negative correlation between pairwise relatedness and spatial distance. Even though animals are closer related to those of their breeding site population (i. e. Stream/Pond) it is impossible to correctly allocate them by the genotype to their source population. The mean pairwise relatedness was stronger for the pond than for the stream breeders. Our results may point to the fact that the fire salamander does not migrate far from their spawning site and that they prefer to breed with other individuals from their patch, therefore they are closer related within than between the groups. Our study had several limitations: in terms of features of

the landscape, physical barriers, like the stone wall from the “Lainzer Tiergarten” north of our study site, may obstruct dispersal and gene flow. This barrier did not affect our population, as both study areas are inside of the wall. According to Hamilton (2009) spatial clustering of individuals might result in mating that is not random throughout a population. Our study area is open for colonization by other fire salamander to the West, therefore gene flow is more probable to occur in the West (Zutz, 2012). This could be the cause for the higher allele frequency at the stream site in the East. Our results may also be partially caused by the lower number of sampling sites compared to Steinfarz *et al*. (2007), who sampled 33 locations in total, as well as by the close proximity of the two investigated locations in our study. Additionally Steinfarz *et al.* (2007) did not use microsatellite analysis, but also mitochondrial D-loop analysis and common-environmental experiments. To reliably exclude an effect caused by the relatively small sample size, it would be advisable to consider a larger amount of specimen for adjacent studies. Furthermore our sampling took place over a short time period, which may lead to the assumption that extension of the observation period to several years would allow to detect the beginnings of genetic differentiation. Positive examples of sympatric speciation in literature are relatively uncommon (Bolnick and Fitzpatrick, 2007). Phillimore *et al*. (2008) used simulations and concluded that sympatric speciation in birds is rare. Investigations of island bird species found no conclusive evidence that they originated sympatrically (Coyne and Price, 2000). Only in the study by Friesen *et. al* (2007) they found sympatric speciation in seabirds due to allochrony, which means that two biological entities occur in the same area but not at the same time. Fitzpatrick *et. al* (2008) critically evaluated the ongoing debate over the prevalence and importance of sympatric speciation in nature, describing how different concepts of sympatric speciation imply different criteria for inferring

cases. Even though sympatric speciation may be theoretically plausible the examples known are still mostly limited to plants, insects and fishes (Savolainen *et. al* 2006; Axen *et. al* 2010; Barluenga *et. al* 2006). Our study on *S. salamandra* could not ascertain genetic differentiation and further evidence of sympatric speciation as suggested by Steinfartz (2007). Overall our results lead us to the assumption that reproductive groups and population subdivision among the two population patches may exist, despite little overall genetic differentiation. For further investigations more samples should be collected from the East and according to our present results they should have an even lower allele frequency. Additionally in future studies DNA could be collected from both stream and pond larvae for definite specification of their spawn site, and therefore adult salamanders that may have migrated from one site to the other could be identified.

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Zusammenfassung

Die meisten Tierpopulationen in freier Wildbahn zeigen eine geklumpte Verteilung. Da Standortdaten einzelner Individuen alleine keine Rückschlüsse auf tatsächliche Muster des Genflusses erlauben, haben molekulare Methoden immer mehr an Bedeutung zugenommen. In dieser Studie untersuchten wir eine Feuersalamanderpopulation aus dem Wienerwald, die eine starke räumliche Strukturierung aufwies. Die jeweiligen Regionen, in denen die Individuen gefunden

wurden unterschieden sich in ihren Möglichkeiten zur Ablegung der Larven: kleine fließende Bäche ("Stream") im Westen und temporäre Teiche ("Pond") im Osten. Ich untersuchte, ob der räumlichen Klumpung der Individuen in diesen zwei Arealen tatsächlich ein Vorhandensein von separaten Fortpflanzungsgruppen zugrunde liegt, indem wir räumliche Positionen und Mikrosatelliten-Genotypen von Feuersalamander aus beiden Gruppen bestimmten und miteinander verglichen.

Ich habe gängige populationsgenetische Methoden, wie die Ermittlung von F_{ST} und F_{IS}, und zusätzlich einen individuell basierten Ansatz verwendet, um Muster von möglicher genetischer Differenzierung zwischen den beiden Gruppen zu untersuchen. Es wurden paarweise räumliche Distanzen und Verwandtschaftskoeffizienten zwischen allen möglichen Paaren von Salamandern analysiert. Während die F_{ST}-Werte keinen Hinweis auf genetische Differenzierung zwischen den Gruppen gaben, zeigte der individuell basierte Ansatz, dass die paarweise Verwandtschaft stark negativ mit dem räumlichen Abstand korrelierte. Die Verwandtschaft war signifikant höher innerhalb einer Gruppe, als zwischen den Gruppen. Dieser Effekt war stärker für die "Pond" als für die "Stream" Individuen. Unsere Ergebnisse zeigen, dass es bei der Untersuchung von genetischer Differenzierung die Wahl der zugrunde liegenden Methode und deren Auflösungspotential von substanzieller Bedeutung ist.

Curriculum vitae

Name: Judith Bürgler

Education:

1991 - 1994	Primary school, VS Sankt Johann im Pongau
1994 - 2002	Gymnasium, BORG Sankt Johann im Pongau
2003 - 2007	Vetmed Uni Vienna
2007 - 2010	University of Vienna, Bachelorstudium Zoology
2010 - 2014	University of Vienna, Masterstudium Zoology

Excursions and field work:

	Praktikum Tiere im Zoo, Bachelorthesis
	Praktikum Alpenexkursion
	Praktikum Zoologisches Labor
	Praktikum Populationbiologie heimischer Amphibien
2011	wissenschaftliche Assistentin am Ludwig Boltzmann Institut für experimentelle und klinische Traumatologie
2012 - 2013	Tierarztassistentin
2013 - present	Account Manager VIRBAC

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