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Annual report of the project:

"Development and constancy of the markings in *Neurergus kaiseri*"

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Introduction:

Neurergus kaiseri is a newt endemic to Iran that, along with *N. derjugini*, *N. microspilotus*, *N. strauchi* and *N. crocatus*, forms the genus *Neurergus* in the family Salamandridae.

K. P. SCHMIDT, 1952 was the first to describe the species.

The external characteristics distinguishing the species from other members of the genus are its relatively small body size (maximum total length 12-14 cm) and its typical black-and-white pattern. It is the only urodelean species known to have a high proportion of pure white coloration. This may represent an adaptation to the strong solar radiation at its breeding sites (SCHULTSCHIK, STEINFARTZ, 1996). The red-orange components of the coloration vary in their intensity depending on individual, age, season and diet.
The terra typica are the streams around the village Shah-Bazan in the southern foothills of the Zagros Mountains in the province Lurestan in the Islamic Republic Iran.
Its entire range is probably very localized and remains mostly unexplored. Little is known about the life habits of this newt in its natural habitat. The animals probably spend most of the year in the highly branched subterranean cave system of the Zagros karst in order to avoid the extreme surface temperatures (0° - 40°C) and the aridity during most of the year.
Due to its high adaptability, keeping this species does not present major technical challenges: it is one of the easiest species to keep in terraria. The animals can be fed with various bloodworms, earthworms, Artemia, Daphnia, Drosophila and other commonly available food items. As opposed to its sister species in the genus, *N. kaiseri* also accepts non-living food (pellets), pointing to an olfactory orientation during feeding. This is evidence for a troglophilic life habit.

Development of the project:

This project was designed to examine the development of the markings during the body growth of the larvae and juveniles. Another aim was to determine whether and to what degree the markings of an individual remain constant over lengthier periods.

The investigations were initiated on 26.04.2010. The study was initially restricted to one year. During this period, the animals were to be photographed in regular intervals.
The impetus for conducting this research was the fact that *N. kaiseri* was listed in Appendix I of the Convention in the Trade of Endangered Species (CITES) on 23.06.2010. Accordingly, the trade in this species underlies CITES conditions, which require that individual specimens be individually recognizable.
As invasive tagging methods such as microchips have potential animal welfare implications in animals of this size range, the task was to find a practicable, technically simple alternative (HENLE et al. 1997). The present study was designed to examine the degree to which the well-recognizable and distinct markings of *N. kaiseri* can be used as a feature to distinguish individual animals.
In many other species, for example *T. alpestris*, *T. dobrogicus*, *T. cristatus* and *P. fuscus*, numerous studies have demonstrated the constancy of the markings as an individual identification over longer periods of time (JEHLE, 1997; HENLE et al., 1997; MATTHÈ et al., 2008).

Material and Methods:

Thirty-six larvae of the species *N. kaiseri* were available. The animals stem from three different captive breeding stocks from Germany, the Czech Republic and Austria.
Metamorphosis in the larval stock from WEGMANN started on 08.03.2010 (F2/2010). The larvae from CURIK first metamorphosed on 31.01.2010 (F3/2010) and those from KLIMA beginning in late December (F1/2010). The parents of the WEGMANN larvae were F1/2007, and those of the CURIK larvae F2/2007. The parents of the KLIMA larvae came from the legal animal trade prior to 2010. Thus, at the beginning of the investigations the larvae were aged 1 month (Box A), somewhat over 2 months (Box B) and approx. 3.5 months (Box C).
The larvae were held in groups of 12 animals each in three study boxes containing 24 l of water each. The water was cleaned and kept in circulation using motor-driven internal filters. Every week, about 1/3 of the volume was exchanged with fresh water. Vienna tap water with a total hardness of 12-14° dGH and a pH-value of slightly over 7.0 was used. Each individual was kept in a small, numbered, individual box (14x7x5 cm) that was open to the surrounding water via mesh-covered openings. This ensured equal conditions for all larvae.

Beyond the boxes for the study specimens, two additional, identical boxes housed comparison individuals. These individuals were raised without the experimental system in order to determine whether the technical set-up influenced the newt development and perhaps the markings (Fig. 1).

Abb.1: Experimental set-up for the 36 larvae in the containers "A", "B" and "C" as well as the comparison individuals in boxes "1" and "2".

All 5 boxes were in the same room under similar conditions (temperature range from 16°C to 25°C over the course of the year). Care was taken to maintain a natural photoperiod. The same food types were available ad libidum for every animal (bloodworms, Tubifex, Daphnia, enchytraeids).

One problem that arose after metamorphosis was that the newts began to escape from their individual boxes. Therefore, at that point (the time differed depending on the individual), the animals were kept in groups of five in a common 24 l box without partitions (Table 1).

Box Nr.	number of animals	housed animals [Nr.]
1	5	4, 27, 38, 40, 41
2	5	9, 26, 30, 36, 39
3	5	12, 13, 25, 32, 35
4	5	2, 5, 8, 10, 15
5	6	1, 3, 6, 7, 11, 43
6	5	14, 16, 17, 18, 19
7	6	20, 21, 22, 23, 24, 42

Table 1: The post-metamorphosis grouping of the individually numbered animals in the 7 boxes (24 l volume each).

The approach required that the markings of each individual animal be examined in defined intervals. Photodocumentation proved to be the optimal method.
At intervals of 7 days, each specimen was photographed from directly above (dorsal perspective) three times in succession. This triplicate effort was necessary because, to avoid disturbance, the animals could not be immobilized. Thus, not all photographs were fully usable. The series of 3 photos per animal always yielded at least one that was optimal for further processing.
The photographic procedure involved removing the animals from their containers once a week, blotting them lightly with absorbent paper, and placing them individually into a petri dish underlain with millimeter-paper. The photographs were then taken from a fixed tripod on a console.
The photodocumentation required patience because the animals, in particularly those in the larval stage, tended to jam themselves in the edges of the dish with their heads: an undistorted photograph required reducing disturbance to a minimum. The animals were then returned to their respective boxes. An effort was made not to touch the sensitive animals with bare hands and to minimize the stress level to the extent possible.
The original plan to document several body regions was dropped because neither the belly nor the sides showed usable markings.
Over the one-year period, each individual was therefore photographed 156 times, yielding a total of 5631 analyzable photos.

The study refers largely to the head pattern because this region showed the most distinct markings. The dorsal markings are used as an additional identification feature.
At the end of the photoseries, the individual photographs were slightly edited (image sharpness and exposure (maximum 2 settings)) (Software: Photo Impact 6) and then converted into black and white with the software program Salamacula v0.9b. This procedure was necessary because only the black and/or white parts of the markings were analyzed. The red-orange color components were filtered out because they depend on the animals' constitution and on various external factors and are therefore not the primary focus of the investigation.
The software ("Salamacula v0.9b") was adapted for this study. Beyond filtering out the color, it was also possible to calculate the number of black and white components in the markings as well as the percent difference between the single photographs of one and the same individual; these could then be set in relation to photos of different animals, helping to determine the relevance of the documentation.

In order to describe the changes in the markings, it was necessary to develop a nomenclature for the individual spots (see page 13).

In principle, *N. kaiseri* uses its pigmentation to develop a black pattern on a white ground color. Based on practical considerations (better descriptive power), however, the present study refers to the white pattern components as “spots”.

Abb. 2: Camera set-up for photodocumentation with a Nikon D3000, AF-S Micro Nikkor 105 mm, Nikon SB-900, 2 Nikon SBR-200, tripod with tripod head Manfrotto 410 (left) and an example of an animal photographed from the dorsal perspective (animal 38).

The relatively long study period and the sensitivity of the small larvae and juveniles were an animal-keeping challenge. With the exception of a small number (5) of metamorphosing young that died during a hot spell in summer, most of the individuals survived the study in good condition.

Specimens 28, 29, 31, 33 and 34 were replaced with animals from the comparison containers (individuals 38, 39, 40, 41, 42).

At the end of the one-year study period, the study specimens proved to have grown somewhat larger than the comparison individuals.

One potential explanation for this size difference is that the individually kept study animals did not experience food competition and were thus able to invest more energy into growth. The comparison individuals, which were held together in boxes without separate containers, had to search for food and probably competed for the best items with the other residents.

Results

The macroscopic comparison of the individual photos of the same animals at 1-week intervals already shows that the greatest changes in the markings took place in the first 6 months of life. Within this timeframe, individual spots that were originally connected to one another could be separated after 5-6 months and show no connections at all (Fig. 3). Another extreme case is the fusion of a multipartite apical spot within only a few months, as seen in specimen 11 (Fig. 4).

Abb. 3: Photodocumentation of specimen 4 with the most conspicuous change in the first 6 months of the study and the loss of the connection between the dextral and sinistral spots and the dorsal line.

Abb. 4: Photodocumentation of specimen 11 with an extreme development of the apical spot: it fused into a unit by the 6th month of the study.

In all 36 investigated newts, the dynamics of the changes of the markings largely ceased after the 6th month. The second half-year was characterized by changes in spot size due to growth of the animals: the proportions of the spots to one another, however, remained distinctly similar.
The onset of the development of the black markings differed in the individual larvae. In some animals (e.g. specimen 4) the markings already began early (within 6 weeks); in others (e.g. specimen 11), certain pigment spots were evident for relatively long (8 weeks) before the final markings emerged.
In most animals, several pigment spots were already present in the early larval stage (4th-5th week of investigation) that could also be detected in the subsequent markings.
The calculation method described below is termed the Salamacula Method. It combines three approaches: the comparison between overlying images, an image transformation and a correlation coefficient procedure. For a detailed description of the method and the program, see (HTTP

follows)

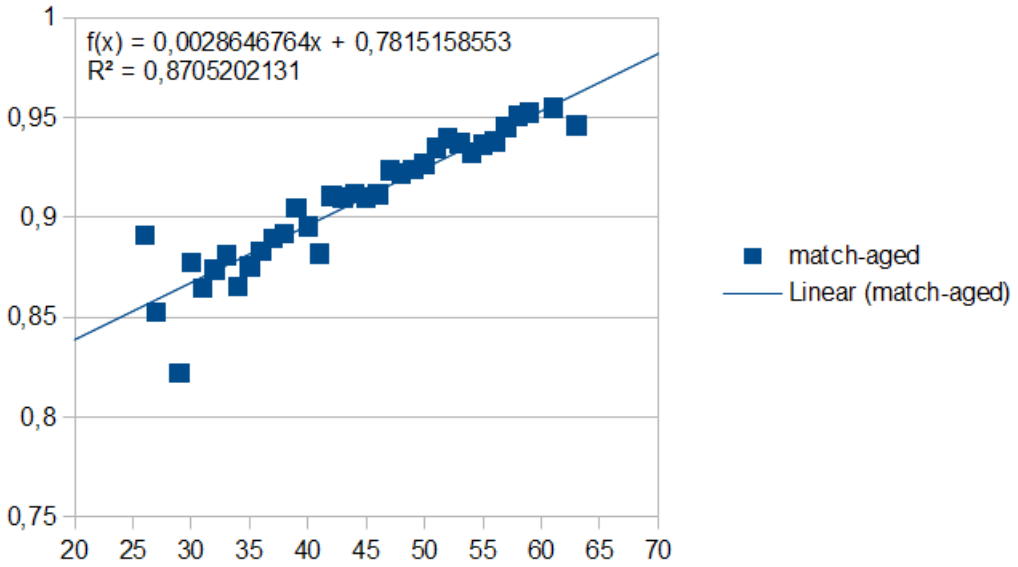

Abb. 5: The x axis describes the age of the salamander in weeks, the y axis the averaged similarity of the patterning (%) at the respective week of age in relation to a 1-year-old salamander.

Calculating the different changes using the Salamacula v0.9.b software revealed an average similarity of 89% between the photo of a 6-month-old versus 12-month-old individual. The average similarity between an approx. 10-month-old and a 12-month-old animal was about 91% and the average similarity between an approx. 11-month-old and a 12-month-old animal was about 95%. These data clearly show a tendency towards decreasing dynamics in the markings with increasing age of the animals (Abb. 5).

Due to this near-constancy after about the 8th month of life, it is entirely feasible to recognize individual animals at this stage based on photographs.

On occasion, however, recognition problems arise due to head markings that are similar between different individuals. In such cases the dorsal markings can be taken as an additional differentiating feature: they represent a sufficient criterion.
In the final phase of the study year (months 11-12) the width of the masticatory (chewing) musculature increased distinctly, whereas snout length, for example, did not recognizably change. Despite this size increase, the relation between the black and white components in the markings

remained largely constant and the individuals were therefore individually recognizable.

Discussion:

This investigation showed that the dorsal head region is the most conclusive with regard to individual differentiation. The study therefore focused on this area based on the distinctive changes there. The dorsal patterning was used as a supplementary identification feature.

The results show that the markings reached near-constancy after about the 8th month of life. After this point, photodocumentation is a suitable tool to recognize individual animals. In the event of highly similar head markings, the dorsal markings can be called upon as a supplementary distinguishing character.
During the study the question arose as to whether backcrossing in *N. kaiseri* could standardize the markings to a point that, after several generations, the animals would resemble one another so closely that individual differentiation based on markings would no longer be possible or very difficult. The presumed restricted gene pool of the animals currently in captivity makes it advisable to examine this issue in the future as well.

Despite the questions raised regarding the restricted gene pool, the authors are of the opinion that photodocumentation as outlined and presented here is a suitable tool for individual recognition in the species *N. kaiseri*.

Outlook:

Upon completion of the initially envisaged study year at the end of April 2011, a decision was taken to continue the photodocumentation. The interval between photographs was extended to 1 month. This approach was designed to ensure recognition of any potential later-developing changes in the markings.

Note that, in the macula nomenclature, the definitions of the individual spots refer specifically to the 36 experimental specimens and to the comparative individuals. In the upcoming weeks, this nomenclature will be consolidated and improved based on photos of other animals.
One study on the markings had already been conducted with Schönbrunn Zoo animals (May 2010). That effort documented 57 animals (0,0,8 individuals from 2007/2008; 0,0,10 individuals from 2000; 0,0,39 individuals from 2010). Of these, 5 in particular showed special combinations of markings in the head regions (one animal still had a divided macula apicalis at an age of over 1 year, four animals had 2 additional spots each above the eyes**)**.

A follow-up project is currently in the planning stage. It is designed to describe the potential hereditary nature of the markings in *N. kaiseri*. The duration of this future study will be about 4 years in order to encompass at least 2 subsequent generations.
More details on this project will be made available at the appropriate time.

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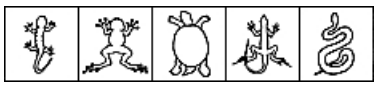

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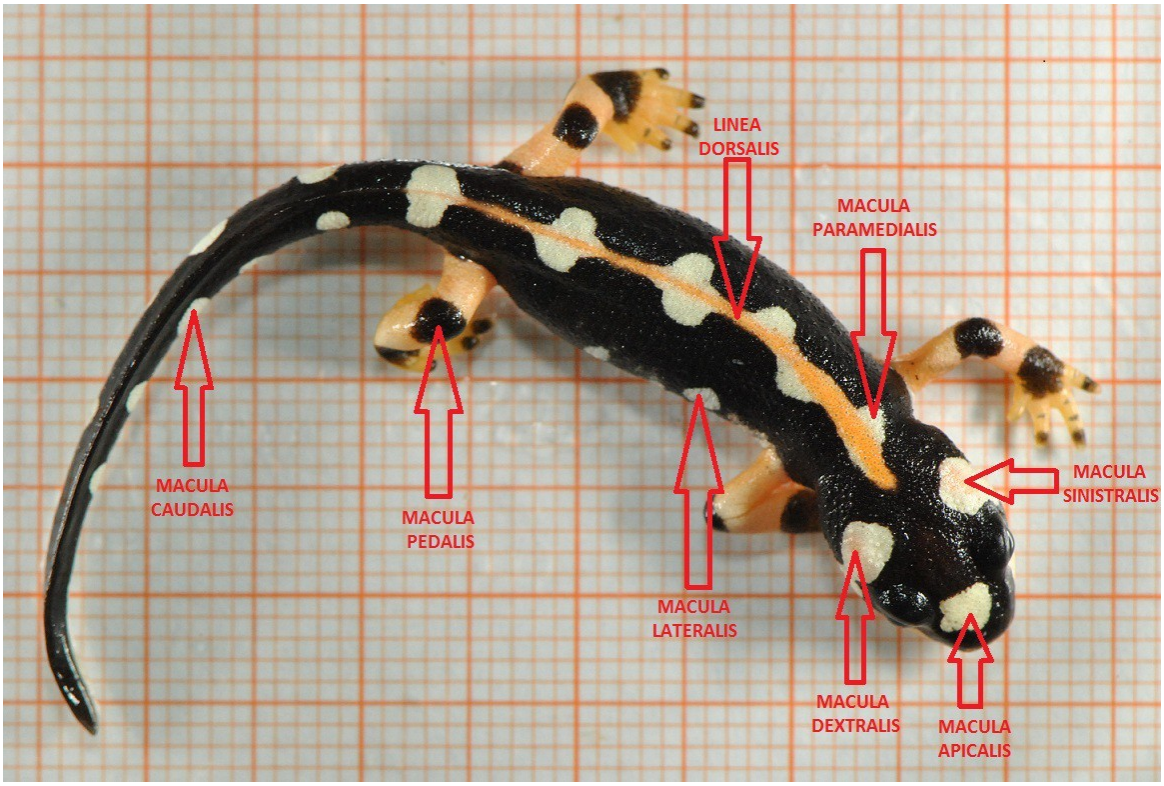
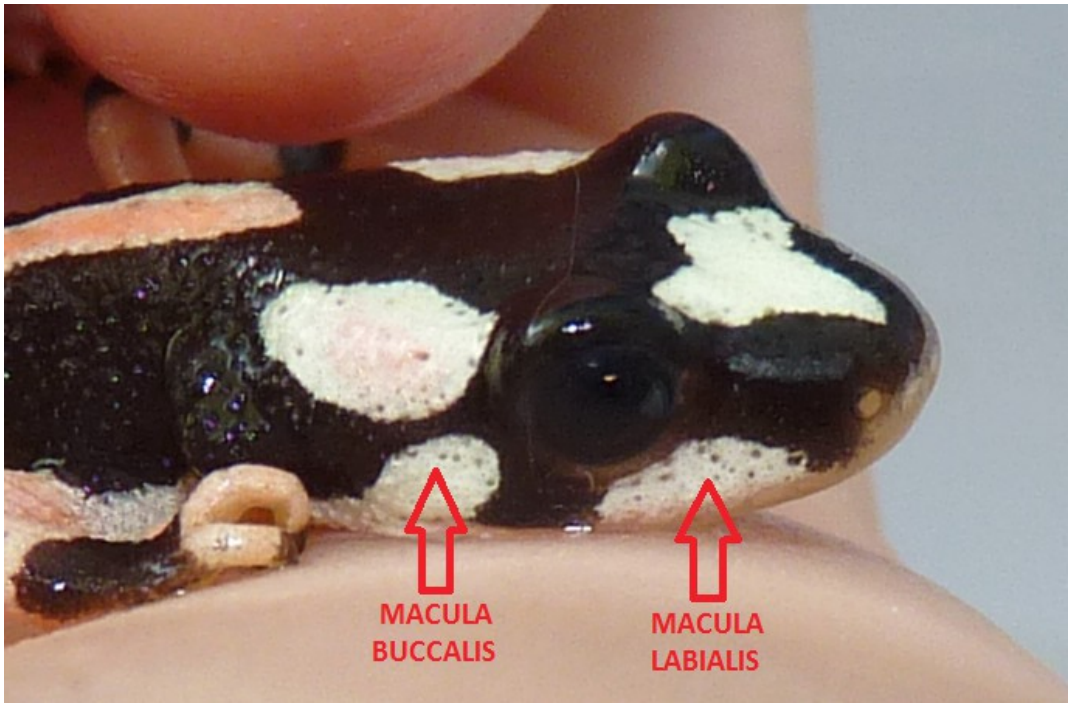
Nomenclature:





Explanation of the nomenclature:

Although *N. kaiseri* markings involve black pigmentation on a white background, the white pattern component is referred to in the following as "spots" based on practical considerations (better descriptiveness).

In most animals the **apical spot** is separated and does not "merge" with the other head spots. Nonetheless, it can take on various shapes and can even be partly divided into several spots (two such animals have been found to date: specimen 32 and an individual from Schönbrunn Zoo from 2009). In contrast, a small number of newts show a connection of the apical spot with other head spots (for example dextral- and/or sinistral spots).

Sinistral and **dextral spots** can develop in different shapes and sizes. They can be separated, fused with one another, or one or both may be connected to the **dorsal line**. Both spots normally extend laterally to the ventral surface. Moreover, they are characterized by a reddish orange coloration in well-nourished individuals.

The **labial spots** are located on both sides of the head above the upper lip. In some cases they can merge under the eye with the sinstral or dextral spot.
The same holds true for the **buccalis spot**, which, however, can extend even further posteriorly. Both spots are often connected with one another.

Paramedian spots are located on both sides of the dorsal line in variable number and shape. They are connected with the dorsal line and can extend as far back as the tail.

The caudal spots begin posteriorly, behind the paramedian spots. They form the lateral markings of the tail and are also present in different numbers and shapes.

The **lateral spots** are located along the sides of the body, typically distinctly delimited from the paramedian spots and positioned lower; they are often connected to the ventral surface.

Pedal spots are present in different numbers and shapes on the limbs of the newts.

To date, four animals have exhibited a special type of spot (Schönbrunn Zoo, animals from 2007/2008). These were more or less distinct and positioned above the eyes and are therefore termed **ocular spots**. More individuals must be examined to determine whether this is an exceptional phenomenon in related individuals.