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Development and constancy of the markings in *Neurergus kaiseri* K. P. SCHMIDT, 1952
(Caudata: Salamandridae)

Entwicklung und Konstanz des Zeichnungsmusters von *Neurergus kaiseri* K. P. SCHMIDT, 1952
(Caudata: Salamandridae)

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KURZFASSUNG

An 36 Larven und Jungtieren von *Neurergus kaiseri* K. P. SCHMIDT, 1952 wurde untersucht, ob und in welchem Ausmaß die individuelle Zeichnung über längere Zeiträume so konstant bleibt, daß sie anstelle potentiell schädlicher, invasiver Markierungsmethoden (z.B. Microchips) zur individuellen Kennzeichnung durch Fotodokumentation verwendet werden kann. Durch die Aufnahme von *N. kaiseri* mit 23.06.2010 in Anhang 1 des Washingtoner Artenschutzabkommens unterliegt das Inverkehrsetzen der Art den CITES-Bedingungen, die zwingend eine individuelle Kennzeichnung des Einzelexemplars vorschreiben.

In der vorliegenden Untersuchung wurden für die Dauer eines Jahres in regelmäßigen Zeitabständen Fotografien der Tiere angefertigt. Ihre Wiedererkennbarkeit auf zeitlich aufeinanderfolgenden Aufnahmen wurde durch die Wahrscheinlichkeit korrekter individueller Zuordnung mithilfe der Bilderkennungssoftware Salamacula überprüft.

ABSTRACT

The changes in the individual colorpattern of 36 larvae and young of *Neurergus kaiseri* K. P. SCHMIDT, 1952, during ontogenesis were studied for the period of one year. Constancy of the markings could warrant reliable individual recognition by photodocumentation in place of invasive tagging methods (e.g., microchipping), which are potentially harmful to small urodelans. *Neurergus kaiseri* was added to Appendix 1 of the "Convention on International Trade in Endangered Species of Wild Fauna and Flora" on June 23, 2010. This is why trading this species is subject to the terms of CITES's regulations, which implies individual recognition of each specimen.

In this study, animals were photographed in regular time intervals during one year. Recognizability of individual newts on consecutive photographs was tested by the probability of correct assignment using the image recognition software Salamacula.

KEYWORDS

Amphibia: Caudata: Salamandridae; *Neurergus kaiseri*, individual recognition, photodocumentation, development of markings, colorpattern, patterning, morphology, ontogenesis

INTRODUCTION

The Lurestan Newt *Neurergus kaiseri* K. P. SCHMIDT, 1952, which is subject to some commercial pet trade, was listed in Appendix I of the "Convention on International Trade in Endangered Species of Wild Fauna and Flora" (CITES) on June 23, 2010 (Anonymous 2010). Accordingly, trading of this species underlies CITES regulations, which requires individual recognition of captive specimens.

Neurergus kaiseri is a newt endemic to the southern Zagros Mountains of western Iran, possibly to be found in adjacent Iraq or Turkey (FROST 2011). The species is distinguished from other members of the genus by its relatively small body size (maximum total length 12-14 cm) and its typical black-and-white pattern (Fig. 1). 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). There are some red-orange components of the coloration that vary in their intensity depending on individual, age, season and diet.

In spring, the animals arrive at streams and creeks to mate and spawn but are rarely

seen the rest of the year, as they probably remain in the highly branched subterranean cave system of the Zagros karst to avoid the extreme surface temperatures (0-40 °C) and the aridity during most of the year.

Due to its adaptability, rearing this species does not present major technical challenges as 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 other species of the genus, *N. kaiseri* also accepts non-living food (pellets), pointing to an olfactory orientation during feeding, evidencing a troglophilic life habit (SCHMIDTLER & SCHMIDTLER 1975; SCHULTSCHIK & STEINFARTZ 1997; SCHULTSCHIK & KARBE 2012).

As invasive tagging methods, such as microchips, have potential animal welfare implications (ARNTZEN et al 1999, 2003; SCHLUEPMANN & KUPFER 2009) in animals of this small size (maximum total length 12-14 cm), the task was to find a practical, yet technically simple alternative among the methods known (e.g., ELMBERG 1989; SINSCH 1992; SEIDEL et al 1996; HENLE et al. 1997; DAVIS & OVASKA 2001; MUTHS 2003; PHILLOTT 2007; HEARD et al 2008; SCHLUEPMANN & KUPFER 2009). In various urodelan species, for example *Ichthyosaura alpestris* (LAURENTI, 1768), *Triturus dobrogicus* (KIRITZESCU, 1903), *Triturus cristatus* (LAURENTI, 1768), *Lissotriton helveticus* (RAZOUMOWSKY, 1789) and *Salamandra salamandr*a (LINNAEUS, 1758) the constancy of the color-pattern markings constitutes a helpful instrument for individual identification over longer periods of time (HAGSTRÖM 1973; HONEGGER 1979; GLANDT 1980; ABBUEHL & DURRER 1993; JEHLE 1997; HENLE et al. 1997; CARAFA & BIONDI 2004; MATTHÉ et al. 2008; COSTA et al. 2009). To determine whether the dorsal patterns of *N. kaiseri* individuals are sufficiently stable and unique to constitute a practical instrument for individual recognition, this project focused on the development of the markings during the period of body growth of the larvae and juveniles. Particular aim was to determine (i) whether or not, and if so (ii) from which time (developmental stage) on or (iii) to what degree the markings of an individual remain constant and can be used as a feature to distinguish individual animals, with the help of photodocumentation.

MATERIALS AND METHODS

Study material.- Thirty-six plus 20 (control) larvae of *Neurergus kaiseri* entered the study; dead specimens (n = 6) were replaced with animals from the controls. The animals, representatives of three age classes (viz. 1, 2 and 3.5 months old), stemmed from three different captive breeding stocks from Germany (F2/2010), the Czech Republic (F3/2010) and Austria (F1/2010). Metamorphosis in the larval stocks started on 8 March, 2010 (F2/2010), 31 January, 2010 (F3/2010) and late December, 2009 (F1/2010) (Table 1). Larval staging followed GROSSE (2012).

Study period.- Monitoring the pattern began on April 26, 2010 and ended on May 5, 2011, during which the animals were photographed in regular intervals.

Rearing design.- The legal regulations of trade (CITES), acquisition (certificate), holding and breeding (e.g., temperature, lighting, size of basin, feeding, cleanness) were observed in all points (SCHULTSCHIK & KARBE 2012). The larvae were held in groups of 12 animals each in three initially uncovered study boxes (100 cm x 50 cm x 30 cm) containing 24 L of water each. 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). (Table 2). 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. Within the above boxes, each individual was kept in a small, numbered, individual box (14 cm x 7 cm x 5 cm) that was open to the surrounding water via mesh-

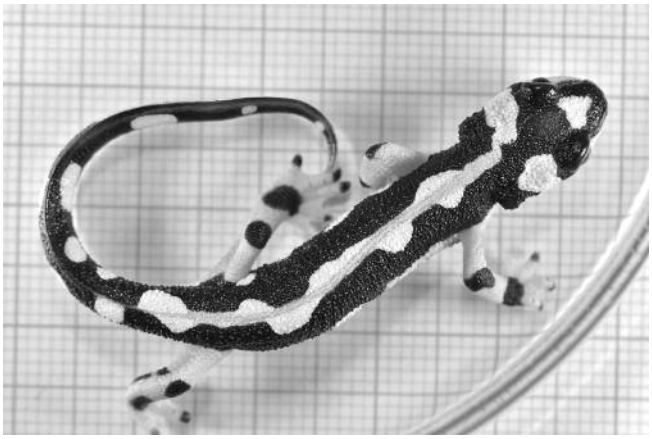


Fig. 1: *Neurergus kaiseri* K. P. SCHMIDT, 1952 (animal #38) in dorsal aspect.
Abb. 1: *Neurergus kaiseri* K. P. SCHMIDT, 1952 (Tier Nr. 38) in Dorsalansicht.

covered openings to avoid inter-individual effects and ensure equal conditions for all larvae.

Beyond the boxes for the study specimens, two additional identical boxes housed control individuals (20 larvae of the F3/2010 stock). These individuals were raised without the experimental compartment system in order to determine whether the experimental setting influenced the newts and perhaps the development of markings (Fig. 2).

All five boxes were placed in the same room under subequal temperature and lighting conditions at a time (temperature range from 16 °C to 25 °C over the course of the year, natural photoperiod, no artificial lighting). Food was available ad libitum for every animal (Chironomidae larvae, *Tubifex*, *Daphnia*, enchytraeids). In a daily morning routine, faeces and untouched food were removed using suction cleaning. After metamorphosis, the newts tried to escape from their individual boxes. Therefore, at that point (the time differed depending on the individual developmental situation), the animals were kept in groups of five in a

Fig. 2: Caging conditions for the 36 larvae in the containers “A”, “B” and “C” as well as the control individuals in boxes “1” and “2”.
Abb. 2: Aufbau zur Haltung der 36 Larven in den Becken “A”, “B” und “C” sowie die Vergleichstiere in den Boxen “1” und “2”.

Fig. 3: Camera set-up for photodocumentation including DSLR camera (Nikon D3000), lens (AF-S Micro Nikkor 105 mm), flash unit (not shown) and tripod with tripod head (Manfrotto 410).

Abb. 3: Aufbau zur Fotodokumentation: Digitale Spiegelreflexkamera (Nikon D3000), Objektiv (AF-S Micro Nikkor 105 mm), Blitzsystem (nicht abgebildet), Stativ mit Kopf (Manfrotto 410).

common 24 L box without partitions under aquatic conditions and the possibility to leave the water by climbing a perforated brick (Table 3).

The long study period of one year and the sensitivity of the small larvae and juveniles were an animal rearing challenge. With the exception of five metamorphosing young that died during a hot spell in the summer of 2010 and one (no. 37) that died two weeks later, the individuals survived the study in good condition. The dead specimens nos. 28, 29, 31, 33, 34 and 37 were replaced with animals from the control group containers (individuals nos. 37, 38, 39, 40, 41 and 42).

At the end of the one-year study, the study specimens had grown to a somewhat larger size than the control individuals. One potential explanation for this difference is that the individually kept study animals did not experience food and spatial competition and were thus able to invest more energy into growth. The control individuals, which were held in groups, had to search for food and probably competed for the best items with their cohabitants.

Photodocumentation.- The study design required the markings of each individual animal to be examined at defined intervals. At intervals of seven days, each specimen was photographed from dorsal in a naturally outstretched position three times in succession. This triplicate effort was adopted to ensure suitable photographs, as the animals were not immobilized so as to avoid disturbance. The series of three photos per animal at any shooting date yielded at least one that was adequate for further processing. The original plan to document several body regions was dropped because neither the belly nor the sides showed usable markings.

The weekly photographing procedure involved removing the animals from their containers, dabbing them softly with absorbent paper, and placing them individually into the dry environment of a petri dish underlain with millimeter-paper. Photographs were taken from a distance of 40 cm using a fixed tripod head (manfrotto 410) to which a DSLR camera (Nikon D3000, lens AF-S micro Nikkor 105 mm) and flashes (Nikon SB-900, 2 Nikon SBR-200) were mounted (Fig. 3). The photodocumentation required patience, because the animals, in particularly those at the larval stage, tended

Table 1: Synopsis of the larval specimens of *Neurergus kaiseri* K. P. SCHMIDT, 1952, available to the present study, including age, number, size and developmental stage (according to GROSSE 2012) when entering the analyses, as well as information about the parental generation.

Tab. 1: Übersicht über die untersuchten Larven von *Neurergus kaiseri* K. P. SCHMIDT, 1952. Angegeben sind neben den Eltern das Alter, die Anzahl und das Entwicklungsstadium der Larven (nach GROSSE 2012) zu Beginn der Analysen.

Age (months) Alter (Monate)	*N*	Beginning of metamorphosis Metamorphosebeginn	Size (cm) Länge (cm)	Developmental stage Entwicklungsstadium	Parents of larvae Eltern der Larven
1	14	28.XII.2009	1.5-2.0	35-36	From legal animal trade prior to 2010
2	14	08.III.2010	2.5-3.0	42-43	F1/2007
3.5	14	31.I.2010	3.0-3.5	44	F2/2007

to jam themselves headlong into the edges of the dish. The individuals were then returned to their respective boxes. An effort was made not to touch the sensitive animals with bare hands to avoid dermal injuries and to minimize the stress level.

Pattern analysis.- The digital color photographs were slightly edited (image sharpness, brightness and contrast, (maximum two graduation marks) (Software: Ulead PhotoImpact 6). Further processing of the images was done with the software program Salamacula ver. 0.9b. Salamacula converted the pictures to images that retained only the originally black and white elements of the color-pattern, whereas the red-orange color components were filtered out. The extent of the red-orange color depended on the animal's constitution and various environmental factors (e.g., the amount of β-Carotene in the insects given as food) and thus, was not in the focus of this investigation. Salamacula counted the number of black and white components in the markings as well as the percent difference between these components on two consecutive photographs of one and the same individual; this information could then be set in relation to photos of different animals, helping to determine the relevance of the documentation.

Table 2: The larval specimens of *Neurergus kaiseri* K. P. SCHMIDT, 1952, available to the present study and their assignation to three boxes (A-C) containing the test individuals and two for the control.

Tab. 2: Die zur Untersuchung verwendeten Larven von *Neurergus kaiseri* K. P. SCHMIDT, 1952, und ihre Verteilung auf drei Boxen (A-C) zur Haltung der Testorganismen und zwei für die Kontrollen.

Box	Number of animals Anzahl Individuen	ID of animals Kennung der Individuen
A	12	1 – 12
B	12	13 – 24
C	12	25 – 36
Control 1	10	27 – 37
Control 2	10	38 – 48

Table 3: The post-metamorphosis grouping of 37 individually numbered *Neurergus kaiseri* K. P. SCHMIDT, 1952, in seven keeping boxes (24 L volume each).

Tab. 3: Die Unterbringung der individuell numerierten *Neurergus kaiseri* K. P. SCHMIDT, 1952 nach der Metamorphose in sieben Hälterungsgefäßen (zu je 24 l Inhalt).

Box number. Becken Nr.	Number of animals Anzahl Individuen	ID of caged animals Kennung der Individuen
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

The procedure was optimized towards processing photographs of the head region. In consecutive pictures of any repeatedly photoraphed individual, the positioning of the head was however not exactly the same, and the lighting and contrast differed as well. Moreover, head size can differ due to variable distance to the camera (variable set-ups or animal movement) or image resolution. Animal growth also plays a role. All these effects had to be considered to obtain a pattern as stable as possible for analysis. Currently, the recognition of the head region is done manually. This calls for entering at least two fixed points that can be clearly identified in every animal and the orientation of which changes only minimally with respect to the pattern. The eyes were chosen for this purpose. The user clicks these on the photo. Using these fixed points, a mathematical procedure automatically rotates and selects the image section (this relies on a vector calculation).

During this process the image is transformed into a black and white image. As in standard computer graphics, the red, green and blue components are weighed according to the perceived brightness (red: 0.299; green: 0.587; blue: 0.114). Thereafter, all image points with brightness values < 50 % are defined as black, the other points as white. The pattern would now be visible but still contains too much "noise" (individual black or white image points in uniformly colored surfaces). These are reduced with a median filter. The filter runs through all image points of the original photograph and examines the points surrounding the respective image point. A filter was selected that examines a surface of 9 x 9 image points. These points are sorted according to brightness. From this sorted list, the brightness of the point in the middle is selected. In the new image, the brightness of that image point is then accepted for the image region examined. The program combines three different methods for the comparison of patterning.

Method A compares the patterns by overlaying them. Here, overlying image points of the same brightness are counted and divided by the total number of image points (24,897 at a resolution of 129 x 193). The result is a value between 0 and 1, reflecting a degree of correspondence ranging from 0 to 100 %. At first glance, this approach appears promising. Experience shows, however, that imprecision, especially in the manual part of the preprocessing, and animal growth strongly influence the calculated values.

Method B is an extension of Method A. As opposed to the static comparison in A, an attempt is made to reduce the effect of both manual processing and animal growth with image transformation (shifting and scaling). The maximum correspondence is approached in a stepwise manner by slightly altering the scaling and shifting. This optimization process represents a type of "Simulated Annealing".

The approach in Method C is to automatically detect the relevant image sections that help to recognize the animals. This builds upon the comparative Method B and the statistical correlation coefficient method. The correlation coefficient indicates the strength of the linear relationship between two characters. In this case those two characters are defined as the correspondence of the image points (at a particular image position of the first pattern) with the comparative pattern as well as the fact that the same animal (assumed value 1) or different animals (value 0) are involved. The input data are therefore the image of the animal's pattern along with information on whether the same animal is involved.

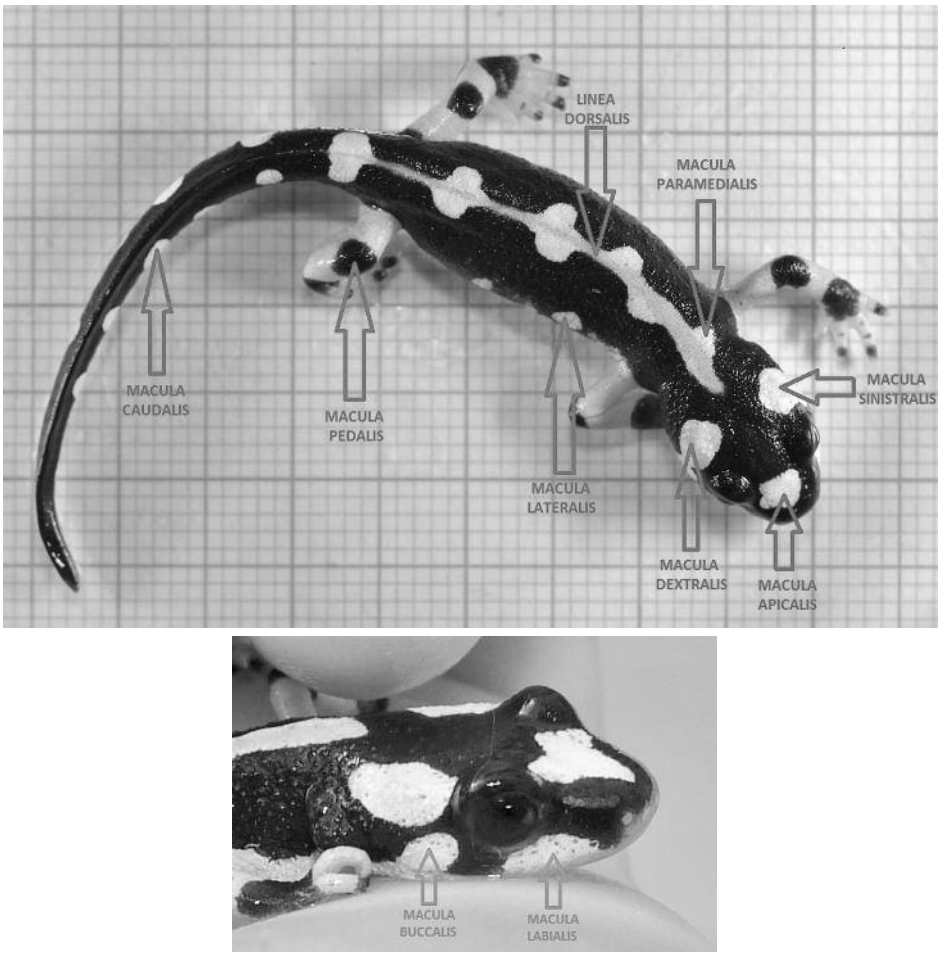
A "map" of the correlation was produced using the correlation coefficients obtained from the image positions. The correlation coefficient ranges between 1.0 (perfect positive correlation) to -1.0 (perfect negative correlation). The higher the absolute value, the stronger the correlation. The closer the value is to 0, the weaker the correlation. Ideally, the correlation coefficients of the image points representing the background should be 0, and in places where distinguishing characters are they should approach 1. This, however, also can yield negative correlation coefficients with relatively small values. The negative correlations mostly pertain to the margins of the animal, indicating that the problem involves (i) different perspectives when taking the photos and (ii) animal growth. The negative correlations are therefore removed (replaced by the value 0). In order to reduce the "background noise" caused by chance relationships, all values are furthermore replaced by their value to the third power ($x = x^3$). This makes very small values even smaller, while larger values remain relatively high. The values are then scaled by dividing them by the maximum value. This yields values between 0 and 1.

RESULTS AND DISCUSSION

Dorsal pattern terminology (Figs. 4, 5).- In order to describe possible changes in the markings, it was necessary to develop a terminology for the individual spots as seen from the newt's dorsal aspect. In principle, *N. kaiseri*'s pigmentation produces a black pattern on a white ground color. Due to practical considerations (increased descriptive power), however, the present study refers to the white pattern components as "spots".

In most animals (91.66 %) the apical spot (*Macula apicalis*) was separated and did not "merge" with the other head spots. Nonetheless, it could take on various shapes and even be divided into several spots (as observed in specimen 32 and an individual from Schönbrunn Zoo, Vienna, from 2009, not included in the materials). Connection of the apical spot with other head spots (for example dextral and/or sinistral spots) was comparatively rare (8.33%).

Sinistral and dextral spots (*Macula sinistra*, *Macula dextra*) could develop to different shapes and sizes. They were separated ($N = 12$), fused with one another ($N =$



Figs. 4-5: Terminology of the markings in *Neurergus kaiseri* K. P. Schmidt, 1952.
Abb. 4-5: Terminologie der Zeichnungselemente bei *Neurergus kaiseri* K. P. Schmidt, 1952.

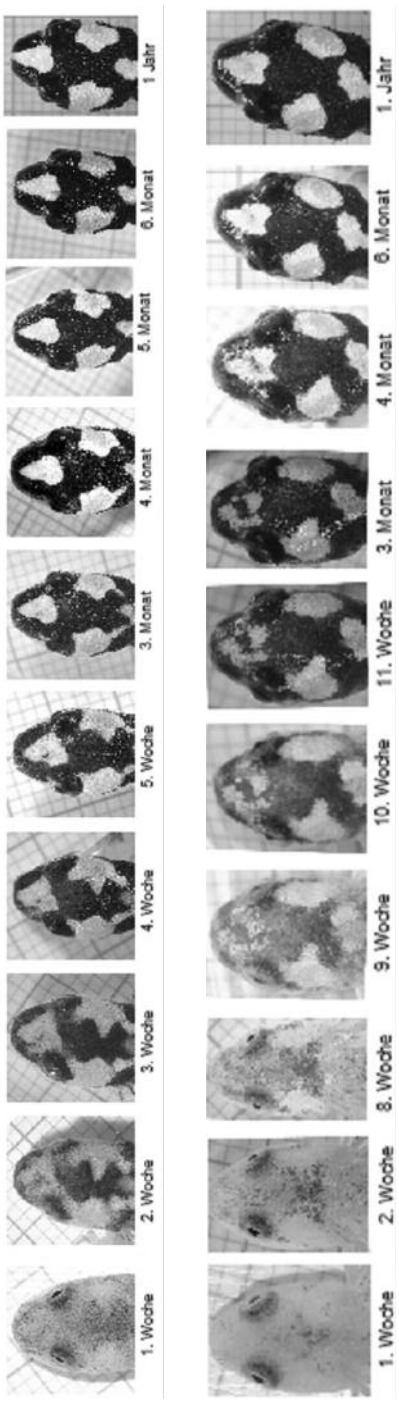
4), or one ($N = 14$) or both ($N = 6$) connected to the dorsal line. Both spots normally extended laterally to the ventral surface and were characterized by reddish orange coloration of the center in well-nourished individuals.

A labial spot (*Macula labialis*) was located on both sides of the head above the upper lip anterior to the eye. In some cases ($N = 7$) it merged with the sinstral or dextral spot under the eye.

Also the buccal spot (*Macula buccalis*) was located on both sides of the head, however, occasionally extended posteriorly. Both spots were often ($N = 21$) connected with one another.

Paramedian spots (*Maculae paramediales*) were located on both sides of the dorsal line in variable number and shape. In all specimens studied they were connected with the dorsal line and could extend as far back as the tail.

The caudal spots (*Maculae caudales*) developed behind the paramedian spots and formed the lateral markings of the tail, where they differed in number and shape.



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The lateral spots (*Maculae laterales*), located along the sides of the body, were typically distinctly delimited from the paramedian spots (N = 34), positioned below them and often connected to the ventral surface.

Pedal spots (*Maculae pedales*) were present in different numbers and shapes on the limbs of the newts.

Four animals, not included in this study (Schönbrunn Zoo, Vienna, from 2007/ 2008), exhibited a special type of spot. These were more or less distinct and positioned above the eyes and therefore termed ocular spots (*Maculae oculares*). More individuals must be examined to determine whether or not this phenomenon is exceptional.

The adspectory comparison of the individual's successive photos shot at one-week intervals already showed that the greatest changes in the markings took place in the first six months of life. Within this developmental period, individual spots that were originally connected to one another could separate and show no connections any more (Fig. 6). Another extreme is the fusion of a multipartite apical spot within only a few months, as seen in specimen 11 (Fig. 7).

In all 36 investigated newts, the dynamics of the changes of the markings largely ceased after the age of six months. The second half-year was characterized by

Fig. 6: Dorsal head region of *Neurergus kaiseri* K. P. SCHMIDT, 1952 (animal #4). Photodocumentation of the specimen which showed the most conspicuous pattern change in the first eight months of life, including the loss of the connection between the dextral and sinistral spots and the dorsal line.

Abb. 6: Dorsale Kopfregion von *Neurergus kaiseri* K. P. SCHMIDT, 1952 (Tier Nr. 4). Die Photodokumentation jenes Tieres mit den prägnantesten Veränderungen in den ersten acht Lebensmonaten zeigt den Verlust der Verbindungen zwischen dem Dextral- und dem Sinistralfleck mit der Dorsallinie.

Fig. 7: Dorsal head region of *Neurergus kaiseri* K. P. SCHMIDT, 1952 (animal #11). Photodocumentation showing an extreme development of the apical spot that consisted of several parts which fused into a unit by the 8th month of life.

Abb. 7: Dorsale Kopfregion von *Neurergus kaiseri* K. P. SCHMIDT, 1952 (Tier Nr. 11). Die Fotodokumentation zeigt eine extreme Entwicklung des Apikalflecks, welcher, aus mehreren Elementen bestehend, bis zum achten Lebensmonat zu einer Einheit verschmolz.

changes in absolute spot size due to growth of the animals; the proportions of the spots relative to one another, however, remained distinctly similar.

Upon hatching, the typical color-pattern has not yet developed. The onset of the development of the black markings differed in the individual larvae. In some animals (e.g., specimen # 4) the formation of definite markings already began at the age of six months (stage 40); in others (e.g., specimen 11), not before the age of eight months (stage 44). In most individuals, several pigment spots already present at early larval stages (from stage 35 on) persisted in the subsequent markings.

Calculating the extent of variation the dorsal color-pattern of each individual underwent, the software revealed an average similarity of approximately 85 % (range = 56.33-92.33, SD = 5.69) between the photos of any individuals at the age (developmental stages in parentheses) of five (stage 38) versus seven (stage 41) months, about 86 % (range = 83.24-91.86, SD = 1.90) at the age of eight (stage 44) versus 10 (stage 45) months, about 89 % (range = 85.04-98.85, SD = 2.52) at the age of approximately eight versus 12 (stage 46) months and about 91 % (range = 87.82-94.90, SD = 1.63) at the age of approximately 10 versus 12 months (N = 36 in all comparisons).

In the final phase of the study year (months 11-12, stage 46) the masticatory musculature increased distinctly along with the width of the head, whereas the proportional length of the snout did not change recognizably. Despite the head's size increase, the spatial relation between the black and white components of its markings remained largely unchanged.

This data clearly shows a tendency towards decreasing dynamics in the formation of markings with increasing age of the animals.

Due to the markings' near-constancy after about eight months of life (minimum average similarity of 85 %), it appears to be feasible to identify individual animals at this age or stage based on photographs.

On occasion (N = 4), however, recognition problems arose due to similar head markings of different individuals. In such cases, the dorsal markings were taken as additional discriminating criteria.

Individual recognition.- The study revealed that the dorsal head region was the most conclusive with regard to individual differentiation. Studies on individual recognition of *Neurergus kaiseri* specimens should therefore focus on this area and the distinctive changes there, with the dorsal patterning as a supplementary identification feature. Ontogenetic development of markings entered a final stage of near-constancy after about the 8th month of life. After this point, photodocumentation was applied as a reliable tool to recognize individual animals.

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.

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