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# DISSERTATION

Titel der Dissertation

## On the performance of the visual system in the nocturnal hunting spider *Cupiennius salei*

Verfasserin

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# Table of contents

# Abstract

The present thesis deals with the visual system of *Cupiennius salei*, a nocturnal hunting spider established as model organism in neuroethology. *Cupiennius* is equipped with eight simple lens eyes that are divided into four pairs according to their position on the carapax. The anterior median eyes are referred to as principal eyes. Two tiny eye muscles attach to the retinae of the principal eyes and shift the eyes' fields of view. The remaining three pairs, the so-called secondary eyes, have stationary retinae and are equipped with a reflecting tapetum.

The eye muscles attaching to the retinae of the principal eyes enhance activity when objects are moving within the visual field of the secondary eyes (Neuhofer et al., 2009). In two of the present studies we investigated the spatial and temporal cut-off frequencies of the secondary eyes. For this purpose, we recorded the eye muscle activity in the principal eyes while visual stimuli were presented to the secondary eyes.

In a first study (Fenk and Schmid, 2010) we determined to what extent the wavelength of a grating can be reduced to still elicit a significant increase in eye muscle activity. The interreceptor angles calculated by Land and Barth (1992) on the basis of anatomical data suggest that spatial resolution in the secondary eyes should be better for vertical gratings than for horizontal ones. We indeed found a difference in the response to moving gratings in different orientations. Vertical gratings elicited a significant response down to a wavelength of 2 deg, horizontal gratings down to 2.7 deg. The difference was, however, less pronounced than suggested by the difference in the corresponding interreceptor angles. Thus we hypothesized that the spiders also respond to temporal intensity modulations. We simulated the intensity modulations in a very simplified model for the movement of the gratings used in our experiments and the results suggest that temporal intensity modulations represent a possible explanation for the behavioural data.

In a subsequent study we could confirm the hypothesis that no directed movement is needed to elicit retinal movements in the spiders. The increase in eye muscle activity as a response to flicker stimuli was then used to estimate the temporal cut-off frequency. In our experiments the cut-off frequency was found to be between 4.3 and 8.6 cycles per second (Fenk and Schmid, submitted). The low behavioural cut-off frequency is well in line with intracellular recordings by Pirhofer-Walzl et al. (2007).

*Cupiennius* was known to be able to hunt successfully crawling and flying prey without any visual input. In a third study we were now able to show that it is possible to elicit attack behaviour with visual stimuli alone, in the absence of any mechanosensory

input. Attack behaviour in these animals might represent an interesting model to study the integration of multi-sensory input in complex behaviour (Fenk et al., 2010b).

A fourth study (Fenk et al., 2010a) deals with the maturation of spider eyes after moulting. We discovered enlarged pigment rings in the eyes of freshly moulted *Cupiennius* leaving a pupil that increases in size in the following days. Using micro CT imaging we could show that the pigment rings cover the part of the cornea that is not yet filled by the growing lens. We suggest that the pigment rings maintain vision in spiderlings after moulting by shielding light rays that would enter beside the lens and degrade the image on the retina.

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# Zusammenfassung

Die vorliegende Arbeit beschäftigt sich mit dem visuellen System von *Cupiennius salei,* einer nachtaktiven Jagdspinne, die als Modellorganismus für die Neuroethologie etabliert ist. Wie die meisten Spinnen besitzt *Cupiennius salei* acht Linsenaugen, die nach ihrer Position am Carapax in vier Paare eingeteilt werden können. Die anterior-median liegenden Augen werden auch Hauptaugen genannt. Die Retinae der Hauptaugen können durch je zwei Muskeln bewegt werden. Alle anderen Augen werden als Nebenaugen zusammengefasst. Sie sind unbeweglich und mit einem licht-reflektierenden Tapetum ausgestattet.

Die Perzeption von Bewegung in den Nebenaugen löst eine Erhöhung der Augenmuskelaktivität in den Hauptaugen aus (Neuhofer et al., 2009). In zwei Studien wurden die Muskelpotentiale in den Hauptaugen aufgezeichnet und eine signifikante Erhöhung der Frequenz als Indikator für visuelle Perzeption in den Nebenaugen herangezogen.

In der ersten Studie (Fenk und Schmid, 2010) wurde die höchste räumliche Frequenz eines Streifenmusters gemessen, für die bei Bewegung der Streifen noch eine signifikante Erhöhung der Muskelaktivität nachweisbar war. Die von Land und Barth (1992) anhand anatomischer Daten berechneten Interrezeptorwinkel legen nahe, dass vertikale Streifen von den Spinnen besser aufgelöst werden als horizontale Streifen. Tatsächlich konnten wir eine Aktivitätserhöhung für vertikale Streifenmuster bis zu einer Wellenlänge von 2° nachweisen, und für horizontale Streifenmuster bis zu 2.7°. Dieser Unterschied ist aber geringer als es die anatomischen Daten erwarten ließen, was zu der Annahme führte, dass die Spinnen auch auf zeitliche Intensitätsmodulationen reagieren. Wir simulierten die Intensitätsschwankungen, die bewegte Streifen in den Photorezeptoren in den zwei Orientierungen auslösen, und konnten zeigen, dass dies eine plausible Erklärung für die Verhaltensdaten liefert.

In einer Folgestudie konnte dann mit Hilfe von Flimmer-Stimuli nachgewiesen werden, dass die Spinnen tatsächlich auf zeitliche Intensitätsmodulationen reagieren. Darauf basierend konnte auch die Flimmerfusionsfrequenz der Spinnen gemessen werden. Bei 4.3 Hz wurde noch eine signifikante Erhöhung der Muskelaktivität registriert, für eine Frequenz von 8.6 Hz konnte keine signifikante Erhöhung festgestellt werden (Fenk und Schmid, submitted). Unsere Daten für die Verhaltensschwelle der Spinnen passen somit sehr gut zu den Werten für die Integrationszeit, welche von Pirhofer-Walzl et al. (2007) in intrazellulären Ableitungen gemessen wurden.

In einer weiteren Studie (Fenk et al., 2010b) wurde erstmals gezeigt, dass *Cupiennius salei* auf bewegte visuelle Stimuli mit abrupten, gezielten Annäherungen reagiert. Dies deutet darauf hin, dass die Spinne trotz ihrer nachtaktiven Lebensweise in der Lage ist, visuelle Informationen für die Jagd zu nutzen. Da *Cupiennius* für ihr ausgezeichnetes mechanosensorisches System bekannt ist, würde sich ihr Jagdverhalten für das Studium multisensorischer Integration bei komplexen Verhaltensweisen anbieten.

In der vierten Studie haben wir die zeitliche Variabilität von Spinnenpupillen beschrieben (Fenk et al., 2010a). Gleich nach der Häutung weisen Spinnen breite Pigmentringe auf, die sich dann im Laufe der Zeit zurückbilden. Mit Hilfe von Micro CT Aufnahmen konnten wir zeigen, dass die Linse nach der Häutung zuerst auf einen kleinen Bereich der Cornea beschränkt ist und die peripheren Bereiche durch die Pigmentringe abgedeckt werden. Wenn sich dann in den folgenden zehn Tagen die Linse auf ihre endgültige Form ausdehnt, gehen die Pigmentringe zurück und der Pupillenradius wächst an. Die Funktion der Pigmentringe könnte es sein, die negativen Auswirkungen der Häutung auf den Sehsinn abzuschwächen, indem sie verhindern, dass Lichtstrahlen, die nicht von der wachsenden Linse auf die Retina gebündelt werden, peripher durch die Cornea in das Auge eintreten.

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# Synopsis

The present studies deal with the visual system of a nocturnal hunting spider. The following chapter provides a brief introduction of the model organism *Cupiennius salei*, focusing on neuro-ethological aspects. The specific aims of the thesis will be listed and finally the results gained will be summarized and discussed.

## Introduction

*Cupiennius salei* (Ctenidae) is a large hunting spider native to Central America. It is most common to Mexico, Guatemala and Honduras, where it is found at sea levels ranging between 200 and 1250 m (Barth, 2002). *Cupiennius* is a strictly nocturnal spider (Barth and Seyfarth, 1979; Seyfarth, 1980) and can be described as a typical sit-and-wait hunter (Melchers, 1967). Adult animals can reach a leg span of more than 10 cm (Barth, 2002).

The spiders retreat during the day, preferably in monocotyledons; moulting, mating and hunting take place during the night (Melchers, 1963; Barth and Seyfarth, 1979). After sunset, at a light intensity of about 20 lx, the spiders leave their hiding place. The animals remain motionless in the retreat's immediate vicinity for about half an hour before searching for an appropriate place to ambush passing prey (Barth and Seyfarth, 1979). At night the spiders are protected from diurnal predators and, perhaps even more importantly, from heat and direct sunlight that could lead to desiccation (Barth, 2002). The greatest distances are travelled by adult males (Fig. 1a) straying at search for females (Fig. 1b) (Schmitt et al., 1990).


**Fig. 1** (a) An adult male and (b) an adult female *Cupiennius salei*.

There are multiple reasons for the choice of *Cupiennius* as a model organism for sensory physiology. Maybe the most important reason is a historical one. Several individuals of the impressive spiders have been transported along with bananas to Munich's largest market hall in the 1960ies and caught the attention of M. Melchers, a PhD-student at that time (Barth, 2002). She cultivated the spiders and studied many aspects concerning the animals' biology (Melchers, 1963; Melchers, 1967). The spiders are bred easily in the laboratory and are not very aggressive. Despite the close relationship to the very poisonous spiders of the genus *Phoneutria* (see e.g., Lucas, 1988), the bite of *Cupiennius salei* is reported to be rather harmless (Barth, 2002). The impressive mechanosensory organs quickly caught the attention of sensory physiologists. *Cupiennius salei* is today certainly the most thoroughly studied spider and probably the only spider species that is considered to be a model organism for neuro-ethology (Uetz and Roberts, 2002). In the past years it has also been established as model organism in the field of evolution and development (for a review see Mc Gregor (2008)).

*Cupiennius salei* is able to accomplish essential behavioural tasks (i.e., prey capture and mating) only using its mechanosensory systems, without any visual input (Melchers, 1967; Hergenröder and Barth, 1983; Barth, 1993). The most important mechanoreceptors involved in the guidance of spider behaviour are *i)* trichobotria, i.e., long hairs that are sensitive to air flow, *ii)* tactile hairs that are sensitive to direct touch, and *iii)* slit sensilla, i.e., sensory organs that can measure strain in the spiders' exocuticula (Barth, 2004). These sophisticated mechanosensory organs are distributed in large numbers over the spiders' body: *Cupiennius* has 90 trichobotria on each leg; several thousands of slit sensilla are embedded in its exoskeleton and the tactile hairs cover more or less the whole spider body, reaching densities of 400 per $mm^2$ (Barth, 2004).

Despite its strictly nocturnal lifestyle and its impressive mechanosensory systems, *Cupiennius* is also equipped with a sophisticated visual system that will be described below.

Spider vision is, as compared to insect vision, a rather neglected field of research and spiders are often thought to have very poor eyesight. This is certainly true for most orb weaving spiders. Hunting spiders, however, are often equipped with excellent eyesight. For a review concerning spider eyes see Land (1985). Spider eyes are simple lens eyes. Most genera have eight eyes which can be divided into four pairs according to their position on the carapax: the anterior median eyes (AME), the anterior lateral eyes (ALE), the posterior median eyes (PME), and the posterior lateral eyes (PLE). The AME are referred to as principal eyes and the three other pairs are summarized as secondary

eyes. Principal and secondary eyes differ in several anatomical, developmental and functional aspects. In the principal eyes the nuclei of the photoreceptor cells are in the centre of the cells and the light absorbing segments lie distal to them, while in the secondary eyes the light absorbing segments are proximal to the cell nuclei. The secondary eyes usually are equipped with a reflecting tapetum whereas the principal eyes lack tapeta but are equipped with a varying number of eye muscles that can move the retina.

Jumping spiders are the family in which visually guided behaviour is most obvious. Spatial resolution in *Portia* was found to be 2.4 min of arc (Williams and McIntyre, 1980) which is half way between the resolution record in insects (14.4 min in the dragonfly *Aeschna*) and the cone spacing in humans (Land, 1985). Such a high acuity can be achieved because the optical cut-off frequency, limited by diffraction at the pupil, is higher in lens eyes than in the facets of insects. *Portia* is the only spider known to have an actual acuity in its principal eyes that approaches the diffraction limit in its lenses (Land, 1985). *Dinopis*, a nocturnal net casting spider, was shown to have a graded refractive index in its large lenses reducing spherical aberration (Blest and Land, 1977). The spiders are extremely light sensitive with a half-maximum response of the photoreceptor cells achieved at light intensities midway between star light and moon light (Laughlin et al., 1980).

The visual system of *Cupiennius salei* has been investigated by Land and Barth (1992). The lenses were found to produce images of good quality on the retinae and it is the coarse receptor mosaic that limits spatial resolution in these animals. In the secondary eyes the photoreceptor cells are arranged in rows within the reflecting tapeta. The interreceptor angles along such a row are smaller than normal to it. The smallest interreceptor angles are around 1 deg and were measured along tapetal rows in the PME and PLE. In the AME interreceptor angles are about 3 deg. The authors also determined the F-number (ratio of the focal length to pupil diameter) of the lenses which ranged between 0.58 and 0.74, implying very bright images (Land and Barth, 1992).

Electroretinograms (ERG) suggested an absolute corneal illuminance threshold for white light of 0.0001 - 0.001 lx (Barth et al., 1993), confirming the assumed high light sensitivity of the spiders' visual system. The threshold was 1 - 2 log units lower than the lowest intensity that could be measured with the luxmeter used by the authors. The receptive spectrum has its maximum at about 450 - 550 nm and drops to zero for wavelength larger than 700 nm (Barth et al., 1993). A subsequent study identified three

groups of photoreceptor cells whose spectral sensitivity curves peak in the green (520 nm), the blue (480 nm) and the UV (340 nm), respectively (Walla et al., 1996).

In *Cupiennius*, a substantial daily microvilli turnover can be observed. The microvillar surface is increased at dusk and is finally ten times larger than during the day before it is again reduced at dawn (Grusch et al., 1997). This should imply a concomitant increase in sensitivity at the spiders' night state. Interestingly, the ERG measurements by Barth et al. (1993) did only reveal an increase in sensitivity for the AME. An analogous situation is reported for the PME in *Dinopis*, where also a daily cycle of the receptive membrane was found (Blest et al., 1978) but no obvious sensitivity change could be revealed using ERG measurements (Laughlin et al., 1980).

The temporal properties of the photoreceptor response to short light pulses were investigated by Pirhofer-Walzl et al. (2007) using intracellular recordings: AME photoreceptors were found to be slightly faster than PME receptors and both eye pairs show an increase in time-to-peak and integration time when dark adapted. The respective integration times of light and of dark adapted PME receptors are 79 ms and 138 ms (Pirhofer-Walzl et al., 2007).

Kaps and Schmid (1996) showed that each principal eye is equipped with a dorsal and a ventral eye muscle. The dorsal muscle is 600 µm long and contains 15-18 striated muscle fibres; the ventral muscle is 650 µm long and consists of 20-22 striated fibres. The dorsal eye muscle arises dorso-laterally from the AME tube and is attached at the dorso-median carapace between the PME. The ventral muscle arises from the ventro-lateral part of the eye tube and is attached at the carapace on the ventral surface of the clypei. The direction of gaze can be altered in the dorso-lateral and in the ventro-lateral direction, and depending on the activity of both eye muscles in any intermediate direction. The action of the eye muscles leads to two different modes of retinal movements: spontaneous microsaccades, generated by the dorsal muscle alone, and induced saccades, that involve the contraction of both muscles. Microsaccades consist in recurring twitches of 2 - 4 deg amplitude (Kaps and Schmid, 1996). These values match well the interreceptor angle measured in the AME by Land and Barth (1992). The microsaccades are thus perfectly suited to prevent an adaptation of the neural image to static objects (Kaps and Schmid, 1996). Induced saccades have amplitudes of up to 15 deg and can be elicited by mechanical or visual stimulation (Kaps and Schmid, 1996; Neuhofer et al., 2009). The arrangement of the muscles is shown in Fig. 2.

The important visual centres in the spiders' brain have been described by Strausfeld and Barth (1993) and Strausfeld et al. (1993) and reveal parallel processing of

visual information. Each of the eight eyes has a separate first- and a separate second-order neuropil, the pathways of the two principal eyes are then merged in a third-order neuropil and the pathways of the six secondary eyes are merged in another third-order neuropil (Strausfeld and Barth, 1993; Strausfeld et al., 1993).

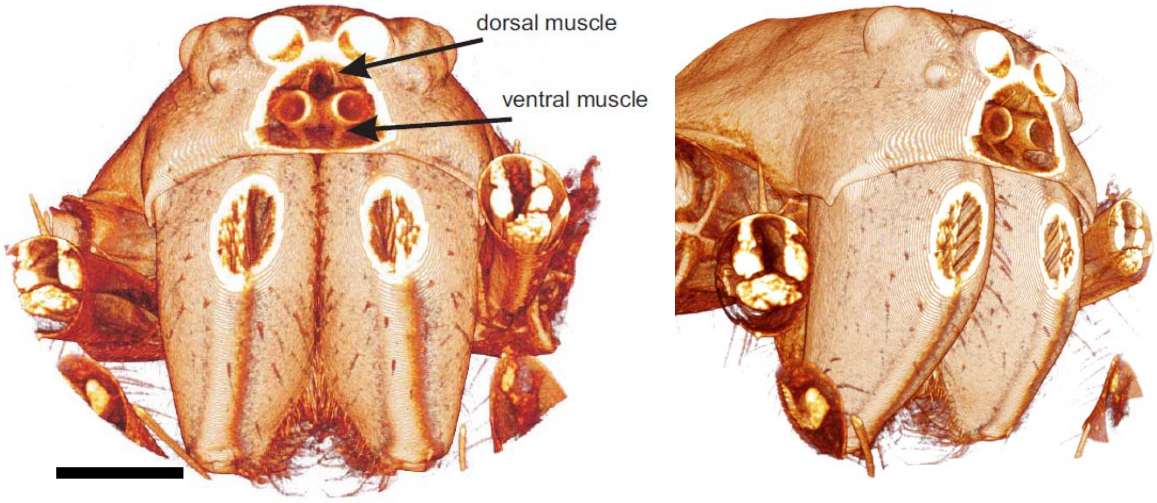


**Fig. 2.** Micro CT reconstruction of the prosoma of a *Cupiennius salei* spiderling. The virtual opening in the reconstruction reveals the arrangement of the dorsal and ventral eye muscles. Scale bar: 1 mm

The anatomical and electrophysiological studies mentioned above strongly suggested that vision is an important sense for this nocturnal hunter, contrasting the "paradoxical absence of visual behaviour" (Land and Barth, 1992, p. 227). Virtually no involvement of vision in any behavioural context could be demonstrated until the late 1990ies. The first manifestation of visual behaviour was that *Cupiennius* changes its locomotion pattern when it is suddenly deprived of visual input (Schmid, 1997). Using light at a spectral composition that is well out of the receptive spectrum of the spiders' photoreceptors (> 950 nm) Schmid observed that *Cupiennius* raises its first pair of legs and probes the surrounds whenever the light visible to the spider is turned off. Interestingly, the second pair of legs does not take over this function, as could be shown in experiments where the first pair was removed (Schmid, 1997). In a subsequent study it was observed that *Cupiennius* approaches appropriate visual targets, which allowed the investigation of the respective role of the principal and secondary eyes in object detection and discrimination (Schmid, 1998). The visual fields of the AME almost completely overlap with the visual fields of the PME (Land and Barth, 1992) which led to the

assumption of a functional specialization. Indeed, while both secondary and principal eyes are sufficient for object detection, the AME were found to be necessary for object discrimination (Schmid, 1998). Neuhofer et al. (2009) showed that eye muscle activity in the AME is only increased when the moving stimuli are presented in the visual field of the secondary eyes. The principal eyes are therefore assumed to be responsible for object discrimination and the secondary eyes for movement detection, as it was already anticipated by Land and Barth (1992). A similar specialization is long known in jumping spiders (Homann, 1928; Land, 1969).

## Specific Aims

This work should address to what extent *Cupiennius* exploits its visual system, i.e., what kind of visual information can actually be perceived by the animals and what it is used for.

Do these nocturnal spiders fully exploit their fine photoreceptor mosaic? And is it possible to reveal differences in the spiders' reaction to gratings of different orientations as predicted by the anatomical data? In psychophysical experiments the eye muscle activity was monitored while the spiders were confronted with moving gratings at different spatial frequencies (Fenk and Schmid, 2010). The difference in the interreceptor angles suggests that vertical gratings can be finer to be properly resolved in the secondary eyes than horizontal gratings (Land and Barth, 1992). We hypothesized that similar differences should be observed in the spiders' response to the onset of moving gratings.

The results of the first study led to the assumption that the spiders also respond to temporal intensity modulations with an increase in eye muscle activity. This was tested in a subsequent study in which the spiders were confronted with flicker stimuli (Fenk and Schmid, submitted). The assumption that no directed movement is needed to increase eye muscle activity could clearly be confirmed. Using flicker stimuli at different temporal frequencies the behavioural temporal cut-off frequency could be determined.

The psychophysical studies indicated that the spiders should be able to detect prey items at any behaviourally relevant distance (at reasonable prey locomotion velocities) and it seemed very unlikely that the spiders would not use this precious sensory input during prey capture. We assumed that, despite the sufficiency of the mechanical senses for successful hunting, also visual input might be integrated in this

behavioural context. We tested this in behavioural experiments where the animals were confronted with moving visual stimuli (Fenk et al., 2010b).

The last study (Fenk et al., 2010a) deals with the visual system of spiders after moulting and addresses questions raised by the observation of peculiar pigment rings in the eyes of *Cupiennius.* The size of the pupil left by the pigment rings was observed to be variable, which was, to our knowledge, not described before. We hypothesized the pigment rings to be connected to the maturation of the eye after ecdysis. This was tested using micro CT images of one spider right after ecdysis and one spider several days later.

## Results and Discussion

A telemetric monitoring of eye muscle activity does certainly not constitute a typical behavioural study. However, retinal movements are the consequence of motor commands processed in higher order brain areas. Thus, a significant increase in muscle activity shows that a certain stimulus has not only been transduced in the receptor cells but that information has travelled along the neural pathways and reached centres responsible for sensory perception, and was there judged to be important enough to elicit an energy-consuming physical response. Such measurements thus provide completely different information about the spiders' sensory world than electrophysiological measurements in receptor cells, and it seems particularly interesting to compare insights gained at the sensory level with such "behavioural" data after information processing.

In the first study we measured the increase in eye muscle activity as a response to the movement of vertical and horizontal gratings at different angular wavelengths presented on a LCD monitor (Fenk and Schmid, 2010). The spiders responded to vertical gratings down to 2 deg wavelengths and to horizontal gratings down to 2.7 deg wavelengths. For the vertical orientation the behavioural cut-off frequency measured corresponded to the frequency of the finest gratings that could theoretically be properly resolved by the spiders' receptor mosaic. The interreceptor angles along tapetal rows are about 1 deg and the finest grating that can thus be properly sampled has a wavelength of 2 deg (Land and Barth, 1992). In the horizontal direction, however, the corresponding interreceptor angles are 2 - 3 deg (Land and Barth, 1992) and a grating at a wavelength of 2.7 deg, which still elicited a significant response, could theoretically not be properly resolved by the photoreceptor mosaic. This led to the assumption that temporal intensity modulations produced by the movement of the gratings are sufficient to elicit retinal

movements. Using a very simplified model we calculated the relative intensity modulations for the finest gratings used in the experiments. The calculations suggested that the differences in the intensity modulations represent a possible explanation for the behavioural data.

In a subsequent study (Fenk and Schmid, submitted) we tested the assumption that no directional motion of objects is necessary to elicit a response in the animals. The animals were confronted with flickering rectangles and we monitored changes in the eye muscle activity upon flicker onset. The spiders responded to slow flicker with very pronounced increases in muscle activity. For flicker frequencies below one cycle per second the averaged response patterns show a rhythmic increase with steep slopes at each intensity change. The response drops quickly for increasing flicker frequencies. The behavioural cut-off in our experiments was between 4.3 and 8.6 cycles per second. Due to the limited brightness and contrast of the computer screen we were not able to determine the maximum cut-off frequency – however, the illuminance used in the experiments matches well the highest light intensities encountered by the animals in their active period. Thus our data might give the order of the biologically relevant temporal cut-off frequency. A temporal resolution of a few cycles per seconds is well in line with the intracellular recordings by Pirhofer-Walzl et al. (2007). The presentation time of one frame at six cycles per second matches the integration time of the photoreceptor cells in light adapted PME.

The psychophysical experiments strongly suggested that this spider could use its visual system in more demanding behavioural tasks than known so far. We could show that it is possible to elicit attack behaviour in *Cupiennius* using slowly moving discs presented on a computer screen (Fenk et al., 2010b). Since the spiders only attack moving objects the presentation of the visual stimuli on a screen was the only possibility to avoid concomitant mechanosensory cues. *Cupiennius* is the first spider for which three different sensory modalities could be demonstrated to trigger attack behaviour independently from each other. Visual cues, air movements, and substrate vibrations have to be integrated in hunting behaviour and this might provide an interesting model to study multisensory integration in the context of complex behaviour. Due to the low behavioural temporal cut-off frequency, one might doubt that the spiders are able to successfully catch fast moving prey using visual cues alone. However, the locomotion patterns of prey contain several phases that could facilitate the detection and localization of prey. A prey item that approaches the spider has a very small angular velocity and would, in the extreme case,

appear just as a spot of increasing size. Any kind of erratic movements, and obviously stop-and-go motion, include moments in which the object's image is less blurred.

The pigment rings discovered in spider eyes could be shown to be related to the maturation of the growing lens after ecdysis (Fenk et al., 2010a). After ecdysis the pupil left by the pigment rings is restricted to the inner fourth of the total projected lens surface. In the following days the pigment ring vanishes. Enhanced post-ecdysal pigment rings were observed in all three spider species examined (*Cupiennius salei, Lycosa tarentula,* and *Heteropoda venatoria*), in both the principal and secondary eyes. We found a rather similar dynamics for the increase of the pupil size in three different age groups examined. Micro CT scans suggest that the pigment rings shield light rays that would pass beside the lens that does not entirely fill out the corneal cup after ecdysis. Such light rays would be expected to be focused behind the retina. Light passing beside the lens would therefore probably degrade the image on the retina and we thus suggest that the pigment rings maintain vision in post-ecdysal spiders by shielding this light.

The interreceptor angles measured by Land and Barth (1992) in the secondary eyes suggest that object localization should be most precise along tapetal rows that are parallel to the spiders' frontal plane. The orientation-dependent spatial cut-off frequency determined in our behavioural experiments suggests that objects moving parallel to the tapetal strips are more easily detected by the animals. This is probably due to the elongated form of the photoreceptors, the receptor subtending smaller angles along tapetal rows than normal to it. This might permit to sample more photons, and thus increase signal-to-noise ratio, while maintaining high precision of spatial information in the spider's principal plane of action. *Cupiennius*' eyes might be tuned to the detection of moving objects at dim light conditions. The visual performance of this nocturnal hunter, showing a relatively good spatial acuity and a relatively low temporal resolution, does well meet the prediction for sedentary animals that are interested in small slowly moving prey (Warrant, 1999).

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# The orientation-dependent visual spatial cut-off frequency in a spider

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## SUMMARY

*Cupiennius salei* (Araneae, Ctenidae) has, like most spiders, eight camera-type eyes. The anterior median eyes are called principal eyes and have a movable retina; all of the other eyes are referred to as secondary eyes and are equipped with a reflecting tapetum. The photoreceptors in the secondary eyes are arranged in rows on the tapetum and the inter-receptor angle along such a row is smaller than normal to it. In this study, the vertical and horizontal spatial cut-off frequencies of moving gratings were measured for the posterior median (PM) eyes, and the data were then compared with the anatomical data reported in the literature. Detection of moving objects in the secondary eyes enhances the eye muscle potential frequency in the principal eyes. We thus recorded the eye muscle activity with a telemetric unit as a monitor for motion detection while moving stimuli – sinusoidally modulated bright and dark stripes – were presented to the PM eyes on a computer screen. A significant increase in the eye muscle activity was measured for gratings at an angular wavelength of 2.0 deg in the vertical orientation and of 2.7 deg in the horizontal direction. In the vertical orientation the critical wavelength is twice the inter-receptor angle; in the horizontal orientation the spiders responded to wavelengths that are smaller than twice the corresponding inter-receptor angle. The cut-off frequency seems thus to be limited by the visual field of the photoreceptors rather than the inter-receptor angle. The relative intensity modulations modelled for the two different grating orientations in single photoreceptor cells were in line with our data.

**KEY WORDS**

*spider eyes, electrophysiology, eye muscles, spatial resolution*

# INTRODUCTION

*Cupiennius salei* (Keyserling, 1877) is a night-active hunting spider from Central America and it is most common in Mexico, Guatemala and Honduras. It prefers to live on monocotyledons, such as banana plants and bromeliads, where it remains sheltered during daytime (Barth et al., 1988b). At dusk the animals begin to hunt for prey and search for mates (Barth and Seyfarth, 1979). The spiders mainly rely on their excellent mechanosensory system in these behavioural contexts as has been shown in numerous studies (e.g. Melchers, 1967; Barth, 1986; Barth et al., 1988a; Baurecht and Barth, 1992; Barth, 1993) and vision seems to play only a minor role. Some manifestations of the behavioural significance of the visual system have been reported by Schmid (Schmid, 1997; Schmid, 1998) and Neuhofer et al. (Neuhofer et al., 2009). The anatomy of the eyes (Land and Barth, 1992; Kaps and Schmid, 1996) and the size and structure of the visual centres in the brain (Strausfeld et al., 1993; Strausfeld and Barth, 1993) suggest an even more important influence of the visual system in at least some behavioural contexts.

*Cupiennius* has, like most spiders, eight simple eyes with a cuticular cornea, a biconvex lens, a vitellar body and a retina. They can be divided into four different pairs according to their position on the carapace. The anterior median eyes (AM) are also called principal eyes, while the anterior lateral (AL), the posterior median (PM) and the posterior lateral (PL) eyes are referred to as secondary eyes.

The astonishingly low *F*-numbers (the ratio of the focal length to the lens diameter) of the spiders' eyes – ranging between 0.74 and 0.58 according to Land and Barth (Land and Barth, 1992) – indicate a high light sensitivity, and indeed the absolute corneal illuminance threshold was found to be well below 0.01 lx (Barth et al., 1993). The human eye has a maximal *F*-number of 2.1 [because the distance between the retina and the optical centre of the lens is 17.1 mm and the diameter of the fully open pupil is 8 mm (Hecht, 2002)] compared with the PM eyes of *C. salei* with an *F*-number of 0.71. This implies that the image of an extended surface at a given luminance on the retina in these eyes is roughly nine times brighter than the image in humans.

The structure of the principal and the secondary eyes differs considerably. All secondary eyes are inverted eyes with the photoreceptor cells turned away from the incident light. They are provided with a reflecting grid-shaped tapetum consisting of several layers of guanine crystals (Fig. 1). The tapetum strips in the PL and PM eyes are roughly orientated parallel to the spiders' longitudinal plane (Land and Barth, 1992). The principal eyes are everted eyes, with the rhabdomeres pointing towards the incident light.

They lack a reflecting tapetum and their retina can be moved by two eye muscles each (Land and Barth, 1992; Kaps and Schmid, 1996).

The optics of the eyes, the quality of the image and the retinal resolution have been investigated by Land and Barth (Land and Barth, 1992). Neither diffraction at the aperture nor the optics of the lens but the fineness of its receptor mosaic limits spatial resolution in *C. salei*. The inter-receptor angles $\Delta\phi$ in rad are calculated as the separation of the receptor centres divided by the focal length. The posterior eyes (PM and PL) have the best resolution with inter-receptor angles of about 1 deg along the rows and 2–3 deg in the vertical direction. The poorest resolution was found in the AL eyes. The angular separation along the rows is 3–4 deg and between the rows is above 9 deg. For the AM eyes an inter-receptor angle of about 3 deg has been measured.

The inter-receptor angles determine the anatomical limit of spatial resolution. A grating can just be properly resolved if the image of one bar falls on a distinct receptor and the image of the next bar on the neighbouring receptor, i.e. the angular period $\lambda$ of the grating is twice the inter-receptor angle ($\lambda=2\cdot\Delta\phi$) (Land and Nilsson, 2002).

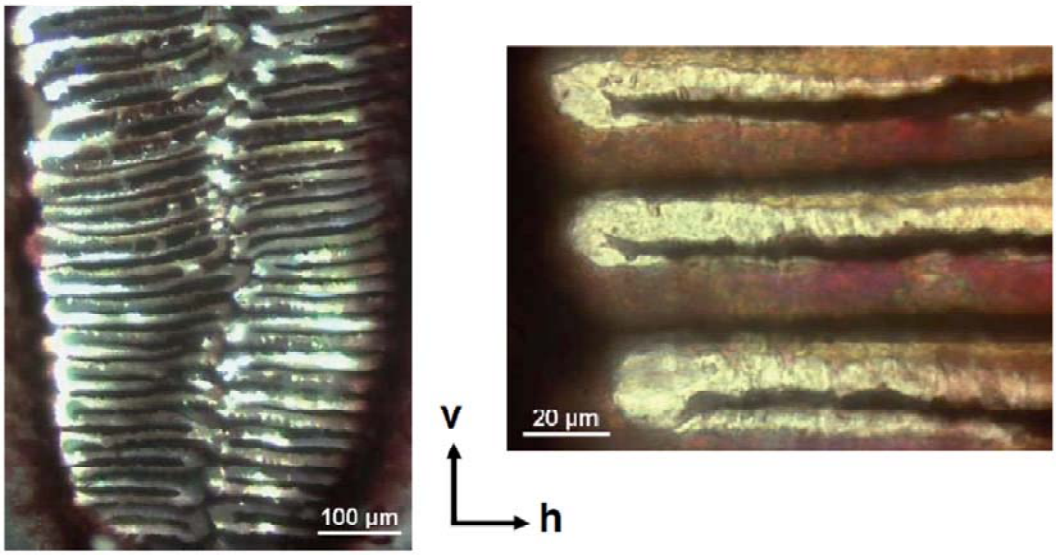


**Fig. 1.** Light microscope micrographs of a freshly removed posterior median (PM) eye retina. On the left side a large part of the grid-shaped tapetum can be seen (using both transmitted and reflected light). The greenish blue gloom is due to guanine crystals reflecting light through the photoreceptors. On the right side (dark field microscopy) only three tapetal strips are shown. One tapetal strip is equipped with two rows of photoreceptors and the axons of the photoreceptors leave the retina through the interjacent slits (Land and Barth, 1992). The arrows indicate the horizontal (h) and vertical (v) axis of the tapetum with respect to the body axis.

The temporal resolution of the photoreceptors was determined by Pirhofer-Walzl et al. using intracellular recordings (Pirhofer-Walzl et al., 2007). The integration time in the dark-adapted state was found to be 138±46 ms in the PM eyes and 86±23 ms in the AM eyes. For light-adapted eyes the authors measured an integration time of 79±17 ms in the PM eyes and of 44±19 ms in the AM eyes (Pirhofer-Walzl et al., 2007).

Kaps and Schmid investigated the structure and function of the eye muscles that move the AM retina (Kaps and Schmid, 1996). Each principal eye is provided with a dorsal muscle that is 600 μm long and consists of 15–18 striated muscle fibres and a ventral muscle that is 650 μm long and consists of 20–22 striated fibres. The ventral muscle inserts at the inner surface of the clypeus and the ventro-lateral surface of the eye cylinder. The dorsal muscle is attached to the exoskeleton in between the two PM eyes and runs to the dorso-lateral surface of the AM eye tube. The passive elasticity of the eye tubes and the eye muscles is assumed to be the counteracting force to the muscle contractions. Two different modes of eye movements have been observed: spontaneous microsaccades are generated by the dorsal muscle only. The muscle activity was shown to be around 12 Hz during the microsaccades, and the retina accomplishes recurring twitches of 2–4 deg in the dorso-median direction. This angle matches the inter-receptor angle in the AM eyes of 3 deg as reported by Land and Barth (Land and Barth, 1992), and it seems indeed reasonable to interpret the microsaccades as a mechanism to prevent the receptor cells from adapting when they are confronted with a static image. Induced saccades, however, are, as shown by Kaps and Schmid (Kaps and Schmid, 1996), generated by both the dorsal and the ventral eye muscles. The amplitude of these movements can go up to 15 deg and their direction can be varied depending on the activity of the two independent eye muscles. The resulting force is the vector sum of the forces produced by the dorsal eye muscle in the dorso-median direction and the ventral muscle in the ventro-median direction. The authors show the relationship between the muscle potentials frequency and the retinal displacement. Saccades are observed in walking animals (Kaps, 1998) and can be induced by mechanical or visual stimulation (Kaps and Schmid, 1996).

Schmid showed that the secondary eyes seem to be responsible for object detection whereas the principal eyes are used for object discrimination (Schmid, 1998). Neuhofer et al. have recently shown that a visual elicitation of the saccades in the eye muscles can only be induced if the secondary eyes are stimulated (Neuhofer et al., 2009). The authors showed that the retinae of the AM eyes move when objects are moving within the visual field of one or more secondary eyes, which suggests that the secondary

eyes alone are responsible for motion perception. This specialisation has already been described for jumping spiders (Homann, 1928; Land, 1969), where the principal eyes that are responsible for pattern recognition show by far the best spatial resolution. Homann compared the principal eyes with the foveal parts and the secondary eyes with the peripheral parts of the human retina (Homann, 1928). Interestingly, in *Cupiennius*, the secondary eyes, the motion-detecting eyes, have the better spatial resolution.

The aim of this study was to determine to what extent these spiders exploit the optics of their eyes. In our experiments we make use of the fact that the perception of moving objects in the secondary eyes enhances the eye muscle activity in the AM eyes. The PM eyes were confronted with movable gratings of variable spatial frequency while the eye muscle activity was monitored *via* a small telemetric unit. The smallest spatial frequency that elicited a significant increase in the eye muscle potential frequency was then compared with the anatomical data reported by Land and Barth (Land and Barth, 1992). We also simulated the intensity changes produced by black and white bars moving relative to a single photoreceptor for the stimuli sizes and velocities used in our experiments by means of a model, which takes the geometry and the integration time of the photoreceptors into account. As the inter-receptor angles as well as the receptors themselves are smaller along the horizontal than the vertical axis, we expected to observe differences in the behavioural responses of the spiders to gratings in different orientations.

# MATERIALS AND METHODS

## Eye muscle potentials

### Animals

Adult female *Cupiennius salei* were used in this study. The spiders were kept in a greenhouse in Vienna at a 12 h:12 h day:night cycle. Relative humidity (70–80%) and temperature (15–28°C) in the stock resemble natural conditions. The spiders were kept separately in glass jars and were fed on flies once a week. 19 spiders were used in the first experimental series, 14 in the second one.

In this study it was necessary to know the position of the PM eye investigated with respect to the stimulus and therefore the spiders had to be tethered. They were cooled down in a refrigerator (at approximately 3°C) and could then be fixed on a

wooden hemisphere using parafilm. The small hairs on the upper side of the prosoma and between the eyes were removed. The telemetric unit was then attached to the spiders' prosoma using beeswax. The reference electrode was inserted laterally into the prosoma, and the measuring electrode just below the PM eyes. The spiders were positioned with their body axis perpendicular to the screen at a distance of 20 cm. The spiders were then rotated 30 deg in the horizontal plane and 65 deg in the vertical plane with the PM eye of interest being the pivot point to ensure that the centre of the stimulus is approximately adjusted to the middle of the visual field of the eye. All but the PM eyes were covered with red acrylic paint.

Care and use of the animals comply with the Austrian animal welfare laws, guidelines and policies.

**Telemetry**

We used a telemetric device as proposed for the wireless transmission of the muscle potentials of a locust by Kutsch et al. (Kutsch et al., 1993), which was recently adapted for spiders (Neuhofer et al., 2009). Using a small, light and extremely sensitive emitter device it is possible to transmit the electric signals generated by the thin eye muscles in *Cupiennius*. The main component is an LC-oscillator circuit, which generates a carrier frequency of about 130 MHz and this carrier frequency is frequency- and amplitude-modulated by the action potentials of the eye muscles. A coil (4 to 5 turns) made of copper wire serves as an inductor. An insulated, very flexible and thin manganin wire is used as a recording electrode (alloy of copper, manganese and nickel; diameter $d$=30 μm; resistance per metre $\rho_l$=628.3 $\Omega m^{-1}$, Isabellenhütte, Dillenburg, Germany). The reference electrode is made of silver wire ($d$=250 μm). The circuit is powered by a silver oxide battery (Renata or Maxell Watch Batteries; 1.55 V) weighing only 270 mg. The battery holder is made of hard PVC, and the mass of the transmitting device plus battery is about 660 mg. The signal could be received by a conventional world receiver (Conrad Voyager RY-630, Conrad Electronics, Hirschau, Germany). It was transmitted through an A/D converter (CED 1401, Science Park Cambridge, UK) to a PC using Spike2 (CED) for data analysis.

**Stimulus**

An LCD monitor (1280 × 1024 pixels, 60 Hz, Belinea, Wittmund, Germany) was used in this study. The stimuli were generated in Matlab (MathWorks, Inc., Natick, MA,

USA), using the Psychophysics Toolbox extensions (Brainard, 1997; Pelli, 1997) and consisted in one-dimensional, sinusoidal, monochromatic gratings together with a transparency mask to prevent fringe effects (Fig. 2A). The spectrum of the screen is shown in Fig. 2B together with the spectral sensitivity of the PM eyes reported by Barth et al. (Barth et al., 1993). 40 lx (Pocket Light Meter, AZ Instrument, 8581, Taichung City, Taiwan) were measured at a distance of 20 cm from the screen showing the presented stimuli.

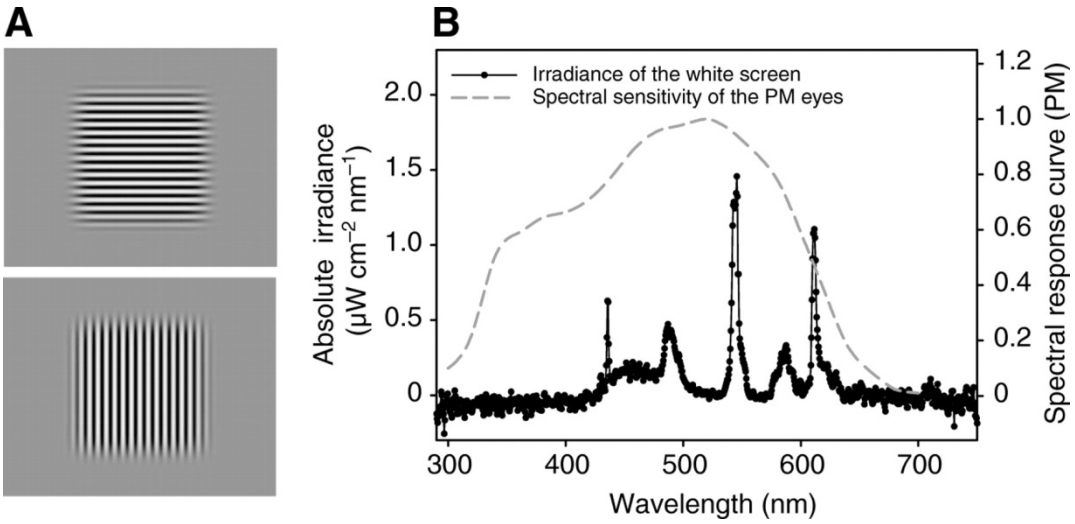


**Fig. 2.** (A) Screen shot of stimuli used in the experiments. This pattern corresponded to an angular wavelength of 3.3 deg for animals at a distance of 20 cm. (B) Spectrum of the light emitted by the LCD monitor used in the experiments together with the sensitivity spectrum of the posterior median (PM) eyes in the day state, modified from Barth et al. (Barth et al., 1993).

In the first series we used eight different spatial frequencies, ranging from 0.117 to 0.667 cycles per degree. The angular wavelength was 8.5 deg for the largest and 1.5 deg for the smallest stimulus. The gratings remained stationary for 50 s and then moved for 5 s at a constant speed (10 deg $s^{-1}$). Each spatial frequency was successively shown four times to the animals; the order in which the spatial frequencies were presented was different for each spider. Then the whole cycle was repeated so that each spider was confronted with each stimulus in all eight times. The movement onset was accompanied by a short trigger signal that could be registered directly with Spike2 together with the muscle potentials.

In the second series only the three gratings with the smallest wavelengths were shown to the animals. Thus, it was possible to confront each spider with the horizontal as well as the vertical orientation to allow a more direct comparison of the reactions. This was not possible in the first series because the presentation of eight different angular

wavelengths in both orientations would have lasted too long and the experiments were limited by the lifetime of the battery. In the second series a constant temporal frequency of 2.5 cycles per second was chosen.

The stimuli sizes have an uncertainty of 0.1 deg mostly due to the positioning of the spiders in front of the screen.

**Analysis**

The eye muscle activity varied greatly during the experiments and we thus compared the mean muscle frequency in the three seconds prior to the movement onset with the frequency in the three seconds after the movement onset for each stimulus. These values were calculated by means of a Spike2 script file and the mean frequency change was determined for the eight stimuli presentations for each spider.

During the experiments the spiders occasionally moved their chelicerae and thus generated signals that were huge compared with the eye muscle potentials. This chelicerae movement was nearly always accompanied by an increase in the frequency of the eye muscle potentials. We therefore excluded stimulus presentations from the analysis whenever such a chelicerae signal was recorded within 25 s before stimulus onset or during the stimulus.

Spiders were excluded from the analysis if more than 10 responses (or more than three for a given stimulus size) were not valid due to chelicerae movements or because of a poor signal-to-noise ratio.

Differences between the mean muscle potential frequency for $N$ spiders before and during stimulation were tested with the Wilcoxon signed-rank test using XLSTAT (Addinsoft, Paris, France). If the frequency was higher during the movement of the gratings, the $P$-value for the one-tailed test was calculated.

## Simulation of the intensity modulation

The temporal intensity modulation at a single model photoreceptor cell produced by a moving rectangular grating was simulated. We considered the size $s$ of the image of a bar on the retina and the receptor size $r$. When the entire receptive area is filled with a white bar the intensity measured by the receptor is defined to be 1, when a dark bar covers the entire receptor the intensity is 0. When the grating moves relative to the retina

the intensity varies over time depending on the ratio of the bar width to the receptor size, on the velocity of the grating and the integration time of the photoreceptor.

For an infinitesimally small integration time only the ratio $r/s$ has to be considered. The maximum intensity differences $\Delta I$ are measured for bar widths $s$ equal to or larger than the receptor, where at a given time the receptor field is entirely filled with the image of a bar or a fraction of it. For decreasing bar widths the maximal intensity differences fall as $\Delta I=2\cdot s/r-1$, and $\Delta I$ obviously equals zero when the stripe width is exactly half the receptor size. Intensity differences increase for further decreasing stripe widths following $\Delta I=-2\cdot s/r+1$, reaching a maximum at $r/s=3$. Considering only odd receptor-to-stripe ratios, where the intensity differences are maximal, the decrease follows $\Delta I=s/r$. If a finite integration time is considered, the maximal intensity differences begin to fall below 1 for bar widths bigger than the receptor size.

The values given above have been calculated assuming a rectangular photoreceptor cross section that is evenly filled with microvilli. In reality the microvilli are restricted to the two vertical boarders of the receptor, and the intensity for vertical bars was therefore calculated as the mean measured by the two microvilli stripes at a given time. The intensity variations for a full cycle were calculated for the wavelengths of the stimuli in our second experimental series. According to the micrographs given by Land and Barth (Land and Barth, 1992), the photoreceptor aspect ratio in the model was chosen to be 1:1.5, and one microvilli stripe was assumed to occupy one-fifth of the total receptor size. The integration time of the photoreceptors in the model was set to 79 ms as reported by Pirhofer-Walzl et al. (Pirhofer-Walzl et al., 2007).

# RESULTS

## Eye muscle potentials

An example of recorded eye muscle action potentials and the frequency increase induced by the movement onset of a grating are shown in Fig. 3. The results of the experiments are summarised in Tables 1 and 2. The mean responses of the spiders, i.e. the difference in the mean muscle potential frequency in the three seconds before and in the three seconds after the onset of the grating movement, are shown in Fig. 4A,B for the two experimental series.

In the first series gratings at angular wavelengths ranging between 8.5 deg and 1.5 deg were presented to the spiders. Nine spiders were shown the vertical gratings and 10 spiders the horizontal gratings. The movement onset of the gratings provoked an increase in the eye muscle potential frequency. The individual responses varied considerably, and the mean frequency increase diminished more or less steadily with decreasing wavelength. The highest mean frequency increase shown by a spider was in the order of 10 Hz and has been measured for the coarsest horizontal grating (8.5 deg). In the vertical subset as well as in the horizontal subset the frequency increases are significant for all stimuli sizes down to 2.7 deg ($0.003<P<0.033$) and not significant for 2.0 deg ($P=0.055$ for vertical gratings, $P=0.480$ for horizontal gratings). The change in frequency is not significant for 1.5 deg ($P=0.953$ for vertical gratings and $P=0.386$ for horizontal gratings). If the two subsets are combined ($N=19$), the frequency increase is significant for all stimuli down to 2.7 deg ($P\leq0.002$); however, the increase for 2.0 deg is still not significant ($P=0.114$). Neither is the change in frequency for 1.5 deg ($P=0.443$). The linear regression (including all data points) has a coefficient of determination of $R=0.97$.

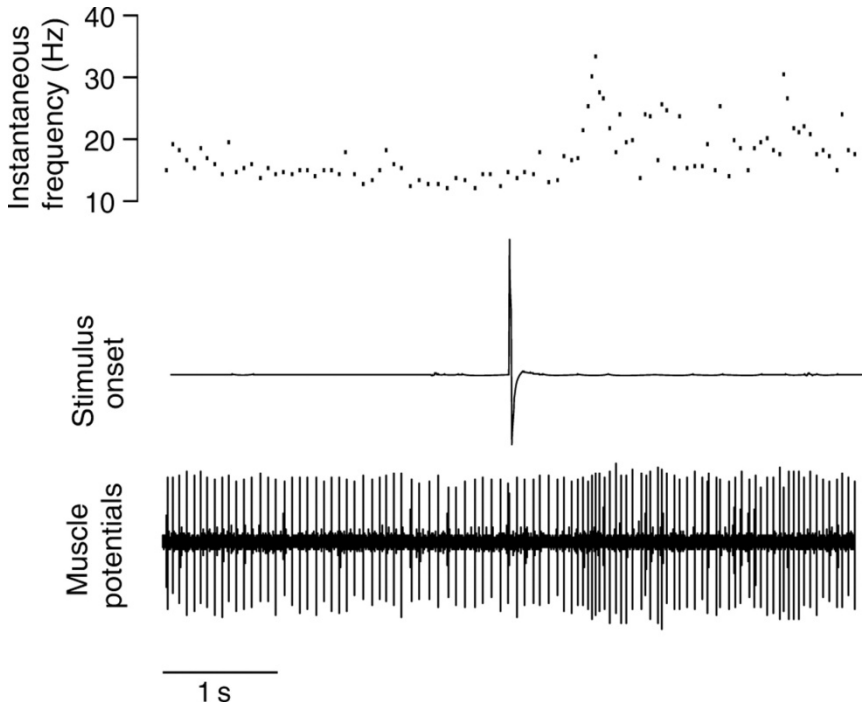


**Fig. 3.** An example for the electrophysiological recordings of the eye muscle action potentials can be seen at the bottom. The middle channel gives the time of the movement onset. Our method is not suited for the measurement of the absolute amplitude; Kaps and Schmid report amplitudes of 50–150 μV (Kaps and Schmid, 1996). At the top the instantaneous frequency of the eye muscle potentials is drawn. The frequency is typically in the order of 15–25 Hz before the grating movement and can go up to 100 Hz.

In the second series (Fig. 4B) we wanted to test if there were significant differences in the response to differently orientated gratings. The spiders (*N*=14) were shown only the stimuli with the smallest three spatial wavelengths of the first series (i.e. 2.7 deg, 2.0 deg and 1.5 deg). This time the gratings were presented at a constant temporal frequency (2.5 cycles per second) and each animal was presented with the horizontal as well as the vertical gratings. Therefore, the reaction of the spiders to gratings of the same spatial frequency but different orientations could directly be compared. The highest mean frequency increases measured for single spiders were in the order of 3 Hz. The response to the 2.7 deg grating was significant for the vertical (*P*=0.005) as well as the horizontal (*P*=0.015) orientation. However, the response to the 2.0 deg grating was significant only for the vertical orientation (*P*=0.013) and not for the horizontal one (*P*=0.801, two-tailed). The difference between the responses to the differently orientated gratings was also significant (*P*=0.035, two-tailed test). As in the previous series the 1.5 deg grating did not elicit a significant change in the eye muscle activity (*P*=0.776 for vertical gratings and *P*=0.826 for horizontal ones).

We thus could show significant responses to moving vertical gratings at angular wavelengths as small as twice the spiders' inter-receptor angle. Horizontal gratings provoked a frequency increase even for wavelengths smaller than twice the inter-receptor angle in this orientation – the inter-receptor angle between tapetum rows is in the order of 2–3 deg (Land and Barth, 1992) whereas gratings at a wavelength of 2.7 deg still elicited a significant response.

**Table 1.** Mean change of the eye muscle activity and the standard error of the mean (s.e.m.) for all spiders of the first series for the vertical (*N*=9) and horizontal subset (*N*=10) measured for the various angular wavelengths of the presented gratings

| Wavelength (deg) | Vertical grating | | Horizontal grating | |
|---|---|---|---|---|
| | Mean +/- s.e.m. (Hz) | P | Mean +/- s.e.m. (Hz) | P |
| 8.5 | 3.0 +/- 0.7 | 0.005 | 3.8 +/- 1.2 | 0.003 |
| 6.1 | 2.0 +/- 0.7 | 0.014 | 1.4 +/- 0.5 | 0.014 |
| 4.8 | 1.2 +/- 0.5 | 0.014 | 1.6 +/- 0.9 | 0.023 |
| 3.9 | 1.2 +/- 0.5 | 0.019 | 1.2 +/- 0.4 | 0.014 |
| 3.3 | 1.5 +/- 0.7 | 0.019 | 1.1 +/- 0.4 | 0.010 |
| 2.7 | 0.5 +/- 0.2 | 0.033 | 1.2 +/- 0.4 | 0.008 |
| 2.0 | 0.7 +/- 0.4 | 0.055 | 0.2 +/- 0.5 | 0.480 |
| 1.5 | 0.1 +/- 0.3 | 0.953 | -0.3 +/- 0.5 | 0.386 |

The P-values were calculated using the Wilcoxon signed-rank test. If the mean difference is positive the value for the one-tailed test is given, if it is negative the two-tailed value is shown.

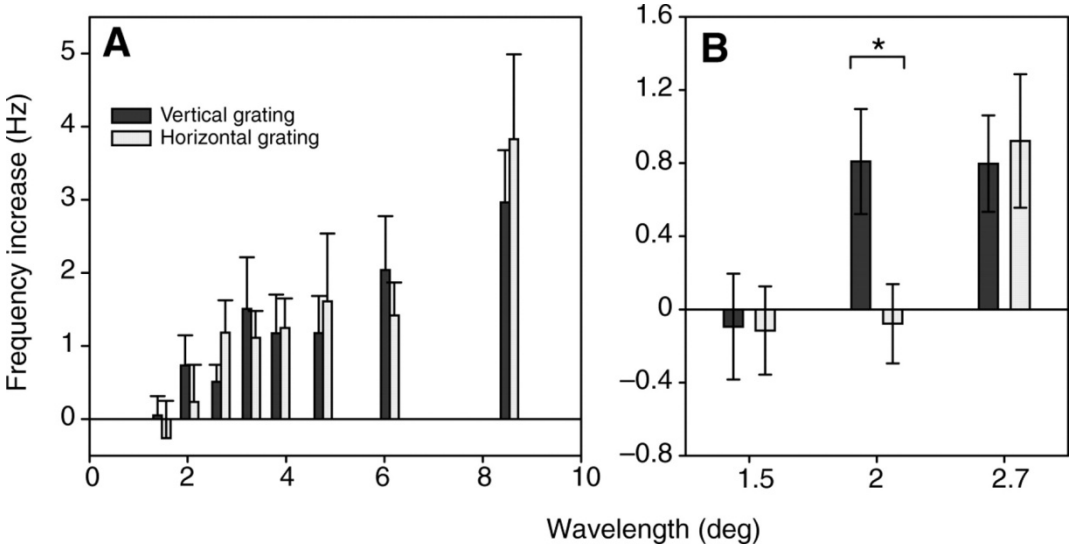


**Fig. 4.** (A) Eye muscle potential frequency increase (mean ± s.e.m.) for eight different angular wavelengths (see Table 1) moving at an angular velocity of 10 deg $s^{-1}$. To one group (*N*=10) the gratings were shown in their horizontal orientation, to the other group (*N*=9) in the vertical orientation. (B) Eye muscle potential frequency increase (mean ± s.e.m.) for angular wavelengths of 1.5 deg, 2.0 deg and 2.7 deg moving at a contrast frequency of 2.5 cycles per second. The increase in muscle potential frequency is significant for 2.7 deg in both orientations. A wavelength of 2.0 deg elicited a significant increase in the vertical but not in the horizontal orientation. The difference between the responses to the two orientations is also significant. The gratings with a wavelength of 1.5 deg did not significantly change the eye muscle frequency.

**Table 2.** Mean change of the eye muscle activity and the standard error of the mean (s.e.m.) for the second series (*N*=14) measured for the various angular wavelengths of the presented gratings

| Visual angle (deg) | Vertical grating | | Horizontal grating | |
|---|---|---|---|---|
| | Mean +/- s.e.m. (Hz) | P | Mean +/- s.e.m. (Hz) | P |
| 1.3 | 0.8 +/- 0.3 | 0.005 | 0.9 +/- 0.4 | 0.015 |
| 1.0 | 0.8 +/- 0.3 | 0.013 | -0.1 +/- 0.2 | 0.801 |
| 0.8 | -0.1 +/- 0.3 | 0.776 | -0.1 +/- 0.2 | 0.826 |

The P-values were calculated using the Wilcoxon signed-rank test. If the mean difference is positive the value for the one-tailed test is given, if it is negative the two-tailed value is shown.

## Simulation of the intensity modulation

Fig. 5A–C shows the simulated light intensity variation measured by a single model PM eye photoreceptor for the angular wavelengths used in the second experimental series (2.7 deg, 2.0 deg and 1.5 deg) during a full cycle. The solid lines show the intensity variation for the horizontal gratings, the broken ones illustrate the intensity variation for the vertical gratings. In Fig. 5D the maximal intensity variations are shown for the three stimuli sizes considering the photoreceptor integration time of 79

ms as reported by Pirhofer-Walzl et al. (Pirhofer-Walzl et al., 2007). The grating velocity in the experiments was 2.5 cycles per second, which corresponds to roughly 0.2 cycles per integration time. Comparing the simulated values with the measured behavioural data we can indeed find a cut-off (indicated by the broken line in Fig. 5D). Above this limit the spiders showed a significant increase (2.7 deg vertical and horizontal gratings, 2.0 deg vertical gratings); the three stimuli for which we calculated lower intensity variations elicited no significant response in our experiments. This could explain why we found a difference between the responses to the different orientations that was less pronounced than expected from the inter-receptor angles. The temporal intensity variation is thus a possible explanation for the gathered data.

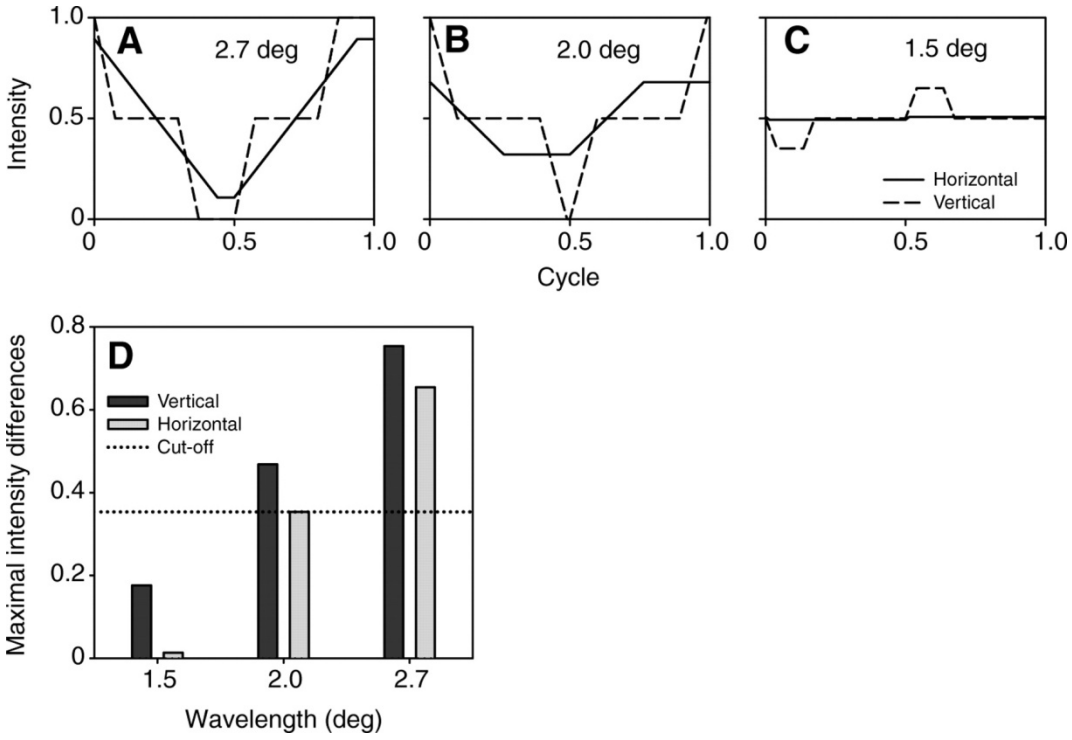


**Fig. 5.** (A–C) Simulated light intensity variation for the three wavelengths presented in the second experimental series (1.5 deg, 2.0 deg and 2.7 deg angular wavelength) measured by a single photoreceptor cell produced by a rectangular grating during a full cycle. The three subfigures show the values calculated for the r/s ratios in the experiments, taking the arrangement of the microvilli in the receptor cells into account. (D) Simulation of the maximum light intensity differences produced by rectangular gratings moving at a velocity of 0.2 cycles per integration time. Intensity changes above the indicated cut-off line (broken line) elicited a significant response in our experiments whereas intensity changes below this limit did not enhance the spiders' eye muscle activity.

# DISCUSSION

In the secondary eyes of *C. salei* the photoreceptor size equals receptor spacing in the horizontal direction (i.e. along a tapetum strip) because the receptors are conjoined but the inter-receptor angle is larger than the angle subtended by the receptors in the vertical direction, i.e. normal to the strips. The finest grating that can be properly resolved by a given retinal mosaic has a spatial period equal to twice the receptor spacing (Land and Nilsson, 2002). In this case the image of one bright or dark bar of the grating falls on a given receptor and the image of the next bar falls on the neighbouring receptor.

In our experiments the finest vertical gratings that elicited a significant response matched exactly this so called Nyquist limit. This means that half the angular wavelength in the vertical orientation of the stimulus equals the inter-receptor angle (and the angle subtended by single receptors) of 1 deg in this orientation. For horizontal gratings we measured significant responses for wavelengths down to 2.7 deg, which is considerably smaller than twice the inter-receptor angle in this orientation. We found a significant difference between the responses to the two different orientations for the condition where the wavelength equals twice the inter-receptor angle in the vertical orientation; here only the vertical gratings provoked a significant activity increase.

There are several possible explanations for these findings. Firstly, the tapetum consists in reality, not only of strips but, as can be seen in Fig. 1, there are regions where the strips turn. These curves are present at the boarders and in the very middle part of the retina, and the inter-receptor angles in these regions are clearly smaller than the angle between the rows. The receptors in the curves could thus enhance acuity in the horizontal direction.

Secondly, it is quite conceivable that the spiders respond to gratings with higher spatial frequencies than the Nyquist limit, because they could still perceive some sort of movement even if the grating is too fine to be properly resolved. The secondary eyes do not have a movable retina, and as *Cupiennius* is a typical sit and wait hunter the retinae of the secondary eyes are often completely stationary and the neural image in these eyes probably adapts (see also Land and Barth, 1992). It is thus thinkable that the spiders might not be able to perceive stationary objects with their secondary eyes but only objects moving in the visual field, producing intensity changes. The secondary eyes could then be imagined as a movement-detecting device using intensity changes over time. For the pure detection of intensity changes produced by small objects, telling the animal that there was something moving, the angle subtended by a receptor rather than the inter-receptor angle

should be the critical factor. Ongoing experiments show that the spiders also respond very well to flicker stimuli (L.M.F. and A.S., unpublished) and this corroborates the assumption that temporal intensity changes trigger the muscular response.

However, even if we consider only the temporal intensity changes at a single receptor, a difference between the two orientations has to be expected because of the aspect ratio of the receptors, which are also larger in the vertical direction than in the horizontal one. We simulated the maximal intensity differences produced by gratings of a given spatial frequency at single model photoreceptors. It is possible to find a limiting intensity change above which the spiders responded to the stimuli and the intensity modulation can thus be considered as a possible explanation for the spiders' performance in our experiments; the difference in the measured spatial cut-off frequency is smaller than the difference between the inter-receptor angles but there is still a difference which can be explained by the geometry of the receptor cells and the resulting difference in the intensity modulations. The temporal intensity changes measured by the photoreceptor cells are the more pronounced, the better the image produced by the lens. This could be one of the reasons why animals would invest in lenses that provide much more detail than the receptor mosaic can resolve.

It might also be interesting to compare our data concerning an arthropod lens eye with the findings reported for insect eyes. Insect optomotor response is direction sensitive but we are unable to make any assumptions concerning the spiders' ability to perceive the direction of motion because there was no difference observed between the reactions to the two different motion directions of vertical gratings and so we can only compare the absolute value of the insect optomotor response with the muscle activity increase in the spider. Due to spatial aliasing the optomotor response of *Drosophila* to moving gratings disappears when the single stripes have a visual angle that exactly equals the inter-receptor angle, and for even smaller bars a response in the opposed direction is observed (Götz, 1964; Götz, 1965). Our data do not suggest spatial aliasing effects in *Cupiennius* similar to those observed in insects because the spiders showed a non-minimum significant response to gratings at wavelengths equal to twice the inter-receptor angle for both grating orientations, where a zero crossing would be predicted.

Our results suggest that spatial summation in subsequent neuronal processes seems not to impair acuity in, at least light-adapted, spiders. In a night-active animal one would then expect an important temporal summation to enhance the reliability of faint images; and indeed Pirhofer-Walzl et al. found integration times of 138 ms for dark-adapted and 79 ms for light-adapted PM eyes using intracellular recordings (Pirhofer-

Walzl et al., 2007). Relatively large integration times and relatively good spatial acuity is what one would predict for sedentary animals interested in small, slowly moving objects (Warrant, 1999).

An inter-receptor angle of 1 deg (Land and Barth, 1992) is impressive for a night-active spider. *Dinopis*, a night-active visual superstar among arthropods, has inter-receptor angles of 1.48 deg in its enormous PM eyes (Blest and Land, 1977), the mean receptor angular sensitivity function having a half width of 2.3 deg (Laughlin et al., 1980). However, *Cupiennius* cannot challenge *Dinopis*' incredible sensitivity. The interommatidial angles of 15 species of bees (day and night-active ones) have been measured by Jander and Jander and were found to range between 1.2 deg and 4.7 deg (Jander and Jander, 2002). Somanathan et al. measured interommatidial angles in a night-active carpenter bee of 0.8 deg in the most acute zone of its visual field (Somanathan et al., 2009). Spatial resolution in *Cupiennius* is thus comparable with the resolution reported for day and night-active bees.

It remains to be shown why this night-active spider invests in such a good eyesight and in what kind of behavioural contexts the visual sense plays an important role.

## ACKNOWLEDGEMENTS

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AL | anterior lateral |
| AM | anterior median |
| PL | posterior lateral |
| PM | posterior median |
| $r$ | receptor size |
| $s$ | size of the image bar |
| $\Delta I$ | intensity differences |
| $\lambda$ | angular period |

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# Flicker-induced eye movements and the behavioural temporal cut-off frequency in a nocturnal spider

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## SUMMARY

We investigated changes of the eye muscle activity in the spider *Cupiennius salei* as a response to temporal intensity modulations. These spiders are known to enhance eye muscle activity in their principal eyes when moving stimuli are detected in the secondary eyes. We monitored the activity of the dorsal eye muscle using a small telemetric unit attached to the spiders' prosoma and confronted the animals to flicker stimuli presented on a CRT monitor. We registered a significant increase in eye muscle activity as response to temporal light intensity modulations which implies that no directed motion is required to trigger the spiders' response. This allowed the determination of the behavioural temporal cut-off frequency. None of the frequencies higher than 8.6 cycles per second and all of the frequencies lower than 4.3 cycles per second did elicit a significant increase in eye muscle activity. A behavioural cut-off frequency of only a few cycles per second is well in line with the temporal properties of the photoreceptor cells determined using intracellular recordings. A relatively low temporal resolution and a relatively high spatial resolution suit well *Cupiennius salei*'s lifestyle as a nocturnal sit-and-wait hunter.

# INTRODUCTION

*Cupiennius salei* (Ctenidae) retreats during daytime and starts to hunt and search for mates at dusk (Barth and Seyfarth, 1979; Seyfarth, 1980). The spider is a sit-and-wait hunter (Melchers, 1967; Barth and Seyfarth, 1979) and extremely polyphageous (Nentwig, 1986). Its mechanosensory systems are well developed and the spiders are able to catch flying or crawling prey without any visual input (Melchers, 1967; Barth and Seyfarth, 1979; Hergenröder and Barth, 1983). Pre-copulatory behaviour involves chemical and vibrational communication and no evidence for visual signalling was found (Barth, 1993). Vision was therefore often assumed to play a minor role, if any, in prey capture and mating behaviour.

However, in spite of the spider's nocturnal lifestyle and its impressive mechanical senses, also the visual system is astoundingly well developed. *Cupiennius* has, like most spiders, eight camera-type eyes. The two anterior median eyes (AME), which are also referred to as principal eyes, are each equipped with a dorsal and a ventral eye muscle that can move the retina (Kaps and Schmid, 1996). The three other eye pairs, the posterior median eyes (PME), the posterior lateral eyes (PLE), and the anterior lateral eyes (ALE), are referred to as secondary eyes. The photoreceptors in those eyes are arranged in rows within light reflecting tapetae and the interreceptor angles along such rows are smaller than normal to it. Land and Barth (1992) investigated the eyes of *Cupiennius salei* and found that the lenses produce images of good quality on the retina. The smallest interreceptor angles are about 0.9-1 deg and were measured in the PME and PLE eyes along tapetal rows (Land and Barth, 1992). This implies a spatial resolution that can challenge typical diurnal insects.

The eyes have also been found to be extremely light sensitive: ERG measurements suggested an absolute corneal illuminance threshold for white light of 0.0001 - 0.001 lx, which was 1 - 2 log units below 0.01 lx, the lowest intensity that could be measured with the luxmeter used by the authors (Barth et al., 1993). These results were confirmed by a more recent study using intracellular recordings (Pirhofer-Walzl et al., 2007). The photoreceptors' spectral range, peaking at 500 - 550 nm, drops to zero for wavelength larger than 700 nm (Barth et al., 1993) and light sensitivity should thus be even better for light conditions encountered in the animals' natural habitat than measured for white light. A substantial microvilli turnover takes place in all eyes, and during night-time the microvillar surface is ten times larger than during the day (Grusch et al., 1997).

A concomitant increase in sensitivity at the spiders' night-state was however only found for the AME (Barth et al., 1993).

The morphological and physiological properties of the eye muscles were described by Kaps and Schmid (1996). The eye cup can be deflected either by the dorsal muscle alone (resulting in microsaccades of about 3 deg amplitude) or by both the dorsal and the ventral muscle (resulting in longer excursions of 4 - 15 deg). The counteracting force to the muscle contractions is presumably the elasticity of the tissues. The authors found a clear correlation between the muscle potential frequency and the deflection of the AME retinae (Kaps and Schmid, 1996).

The elaborate visual system and the importance of the visual centres in the brain (Strausfeld and Barth, 1993; Strausfeld et al., 1993) suggested the significance of the visual sense for these nocturnal spiders, which could be confirmed in several behavioural studies: Schmid (1998) showed that *Cupiennius salei* approaches appropriate visual targets and using twofold choice experiments the different functions of the two sets of eyes could be investigated. The spiders are able to detect visual targets using either the secondary or the principal eyes, but for object discrimination input from the principal eyes is required (Schmid, 1998). The spiders respond to movement presented in the visual field of the secondary eyes with an increase in eye muscle activity in the principal eyes (Neuhofer et al., 2009). Thus, the secondary eyes seem to be responsible for movement detection, while the principal eyes are necessary for object discrimination (Neuhofer et al., 2009). The motion detecting system was very recently shown to be colour blind (Orlando and Schmid, 2011). The field of view of the AME is shifted during locomotion; the spiders enhance eye muscle activity in the ipsilateral eye before turning and thus look in the subsequent walking direction (Schmid and Trischler, 2011). Visual stimulation alone is sufficient to release attack behaviour and this strongly suggests that the spiders are able to use visual cues in the context of hunting behaviour (Fenk et al., 2010b).

To test the spiders' spatial cut-off frequency we recorded the increase of the eye muscle activity as response to moving gratings at different wavelengths (Fenk and Schmid, 2010). The difference between the interreceptor angles along tapetal rows and the interreceptor angles normal to the rows implies an orientation-dependent retinal resolution (Land and Barth, 1992). Our data indeed revealed an orientation-dependent spatial cut-off frequency; the difference between the two orientations was however less pronounced than the difference between the corresponding interreceptor angles. This led to the assumption that the spiders also react to pure temporal intensity modulations and that, consequently, the angles subtended by one photoreceptor in the two orientations are

the limiting factor for the response to moving gratings. A simple simulation of the intensity modulations taking the photoreceptor geometry into account, suggested that this represents a possible explanation for the reaction of the spiders to horizontal gratings that were actually to fine to be properly resolved by the receptor mosaic (Fenk and Schmid, 2010).

In the present study we tested the assumption that temporal intensity modulations lead to significant responses by recording the muscle activity with a telemetric unit while the spiders were confronted to stimuli presented on a CRT screen. Our assumption was clearly confirmed. The activity increases as response to flicker, moreover, allowed the estimation of the behavioural flicker fusion frequency in this nocturnal spider. The values obtained in our experiments are well in line with the integration time measured by Pirhofer-Walzl et al. in intracellular recordings (Pirhofer-Walzl et al., 2007).

## MATERIALS AND METHODS

### Animals

We breed *Cupiennius salei* in a greenhouse where relative humidity (70 - 80%) and temperature (20 - 28°C) resemble the conditions in the spiders' natural habitat. The animals are kept separately in 5 liter glass jars under a 12h:12h day:night cycle and are fed flies (*Calliphora* sp.) once a week. In this study we used 19 adult female spiders. For the experiments the spiders were cooled down and subsequently tethered onto a turnable wooden spherical cap that was connected to a magnetic stand by means of a ball bearing. The legs, pedipalps and chelicerae were fixed with Parafilm bands, the prosoma and opisthosoma were left free. The hairs on the upper part of the prosoma and between the PM eyes were removed before the telemetric unit could be attached to the prosoma using beeswax. The reference electrode was inserted posterio-laterally into the prosoma; the measuring electrode was placed just below a PME. A picture of a tethered spider is shown in Neuhofer et al. (2009). When a sufficiently good signal-to-noise ratio was achieved, the spiders were positioned at a distance of 20 cm from the screen and rotated about 30 deg in the horizontal and in the vertical plane. All but the PME were covered with red acrylic paint.

Post-ecdysal spider eyes show enlarged pigment rings that diminish while the lens is growing (Fenk et al., 2010a). The pigment supposedly shields light rays that would enter the eye beside the growing lens and might maintain vision in post-ecdysal animals.

However, light sensitivity is certainly altered in this state and we thus only used animals that did not show significant rings and could therefore be assumed to have fully developed lenses.

**Stimulus**

The stimuli were generated in MATLAB (MathWorks, Inc., Natick, MA, USA) using the psychophysics toolbox (Brainard, 1997; Pelli, 1997) and were presented on a CRT monitor (800 x 600 pixels, 120 Hz; Sony Trinitron Multiscan 300sf, Tokyo, Japan). The monitor was turned on at least one hour before the experiments started. In the following flicker frequencies will be given in cycles per second (cps).

In the first experimental series, we monitored the response of the animals to bright and dark single step stimuli and slow flicker. In this series the whole screen was covered with a stationary checkerboard during inter-stimulus time; the flicker stimuli were shown in an 18.5 cm x 18.5 cm rectangle in the middle of the checkerboard pattern. The checkerboard pattern had a wavelength of 1 deg which is well below the retinal resolution reported for *Cupiennius salei* (Land and Barth, 1992). We measured the reaction of 7 spiders to the appearance of a dark rectangle, a bright rectangle, flicker starting with a bright rectangle (0.278 cps, 0.554 cps, and 1.11 cps), flicker starting with a dark rectangle (0.278 cps), and a counter-phase flicker (0.554 cps). For the counter-phase flicker the rectangle was divided into two vertical halves and the overall intensity remained constant over time. The stimuli were shown for about 6.3 – 7.2 s, depending on the stimulus type; the inter-stimulus time was 50 s. We pooled for each spider the first five valid measurements, i.e., measurements for which the signal-to-noise ratio was sufficient and where no chelicerae movements have been registered in the 20 s prior to the stimulus onset or within the first 3 s after stimulus onset. The order in which the different stimuli were shown was different for each spider.

In a second series we determined the spiders' behavioural flicker fusion frequency. Here only the 18.5 x 18.5 cm rectangle showed the checkerboard pattern (1 deg wavelength) and the surrounding background was grey. Precise timing was crucial for the correct presentation of high frequency stimuli and the stimuli were therefore chosen to be as simple as possible. To keep processor load (and thus ventilator noise) as constant as possible the checkerboard pattern flickered at the same frequency as the subsequent stimulus. After an inter-stimulus time of approximately 30 - 34 s the bright rectangle appeared in place of the checkerboard pattern and started to flicker at a given frequency for 5.6 - 6.0 s, depending on the flicker frequency. 12 spiders were shown

seven different flicker frequencies ranging between 0.55 cps and 60 cps. Again, the order in which the different stimuli were shown was different for each spider. The given sequence of the seven stimuli was repeatedly shown to the spiders (typically 6 - 8 times) until six valid measurements were recorded for each flicker frequency.

The stimuli were shown at the maximal brightness possible with the monitor used. We measured a luminosity of 56.0 ± 0.5 cd/m$^2$ for the bright rectangles, of 2.15 ± 0.05 cd/m$^2$ for the dark rectangles, and of 28.0 ± 0.5 cd/m$^2$ for the checkerboard pattern (Luminance Meter, LS-100, Konica Minolta, Tokyo, Japan). The asymmetry, caused by the rendering of the checkerboard on the CRT, was thus in the order of 1 cd/m$^2$. The subsequent slight increase in luminance upon flicker onset was not sufficient to enhance eye muscle activity (see results for high flicker frequencies). The contrast of the bright and dark rectangle was 0.93. Screenshots showing the stimuli used in the two experimental series are provided as supplementary material.

**Telemetry**

The activity of the dorsal eye muscles was, as already described in previous studies (Neuhofer et al., 2009; Fenk and Schmid, 2010), recorded with a small telemetric unit. The telemetric device proposed by Kutsch et al. (1993) was adapted for spiders in our group. Its main component is a LC-oscillator circuit that generates a carrier frequency of roughly 130 MHz that is frequency and amplitude modulated by the AME muscle potentials. The signal was recorded using a conventional world receiver (Conrad Voyager RY-630, Conrad Electronics, Hirschau, Germany) that was connected to a PC via an A/D converter (CED 1401, Science Park Cambridge, UK). We registered the muscles activity for data analysis using Spike2 (CED). This setup is not suitable to measure the absolute amplitude of the muscle potentials, which is, however, not needed for our analysis.

**Analysis**

The eye muscle activity shows a great variation and depends on a large number of internal and external factors. The spiders show different resting activities, probably depending on their state of arousal and the activity increases as response to visual stimulation but also as response to mechanical stimuli (Kaps and Schmid, 1996). We thus averaged several stimulus presentations for each individual spider (5 in the first series and 6 in the second one).

To visualize the temporal pattern of the spiders' response we calculated the mean frequency as a function of time for the 35 presentations of the first experimental series showing slow flicker and 72 presentations for the second series used to determine the cut-off frequency. For the calculation of the mean we exported the mean muscle potential frequency (bin size: 0.2 s) with an output sample rate of 100 Hz. To determine the highest flicker frequency that elicited a significant increase we compared the instantaneous muscle potential frequency averaged in the three seconds prior to stimulus onset to the averaged frequency in the three seconds after stimulus onset.

We excluded stimulus presentations when the signal-to-noise ratio was not sufficient or when body and/or chelicerae muscle contractions were recorded within 20 seconds prior to the stimulus presentation or in the first three seconds after stimulus onset.

The significance of the frequency changes were tested for the spiders with the Wilcoxon signed-rank test using MATLAB (N = 7 for the first series, N = 12 for the second series).

# RESULTS

### Single step stimuli and slow flicker

The spiders responded with a pronounced increase in eye muscle activity to slow flicker, to counter-phase flicker, as well as to step stimuli. The mean frequency increase in the three first seconds following the onset of the stimuli compared to the three seconds preceding the stimulus onset ranged between 3.0 Hz and 9.3 Hz. The responses to all 6 stimuli were significant (Table 1). Interestingly, the mean increase elicited by the counter-phase flicker (4.4 ± 1.1 Hz) was roughly half of the increase elicited by the rectangle of the same size that flickered homogeneously at the same frequency (8.9 ± 1.8 Hz). The mean activity increases as response to the two step stimuli (checkerboard to bright and dark rectangle) were in the same order of magnitude (3.0 and 3.6 Hz respectively) and step stimuli elicited a significantly lower activity increase than the flicker stimuli at low frequencies. Fig. 1 shows the temporal pattern of the response to the single step stimuli and to the lowest presented flicker frequency (0.278 cps). The curves represent the arithmetic mean of the mean activity (bin size 0.2 s) for the first five stimulus presentations to all spiders (n = 35). Both the step stimuli and the slow flicker stimuli elicited a mean increase that is steeper for the onset of the dark rectangle than for

the bright one. Pooling the response to the step stimulus and the first intensity change of the slowest flicker (0.278 cps) for the first 0.6 s after stimulus onset, we obtain a slope of approximately 3.0 Hz/s for the change from the checkerboard pattern to a bright rectangle, and about 8.5 Hz/s for the change to a dark rectangle (with $R^2 > 0.95$ for both regressions, N = 7, n = 70).

The rhythmic patterns that are observed for the averaged responses of all spiders (N = 7, n = 35) can also be observed in single stimulus presentations. Fig. 2 shows the response of the spider with the lowest resting activity, where the rhythmic increase can, as a consequence, be most easily observed. This spider has primarily increased muscle activity upon a decrease in light intensity. The pre-stimulus activity was below 10 Hz and frequency was increased up to 75 Hz for the third change to the dark rectangle.

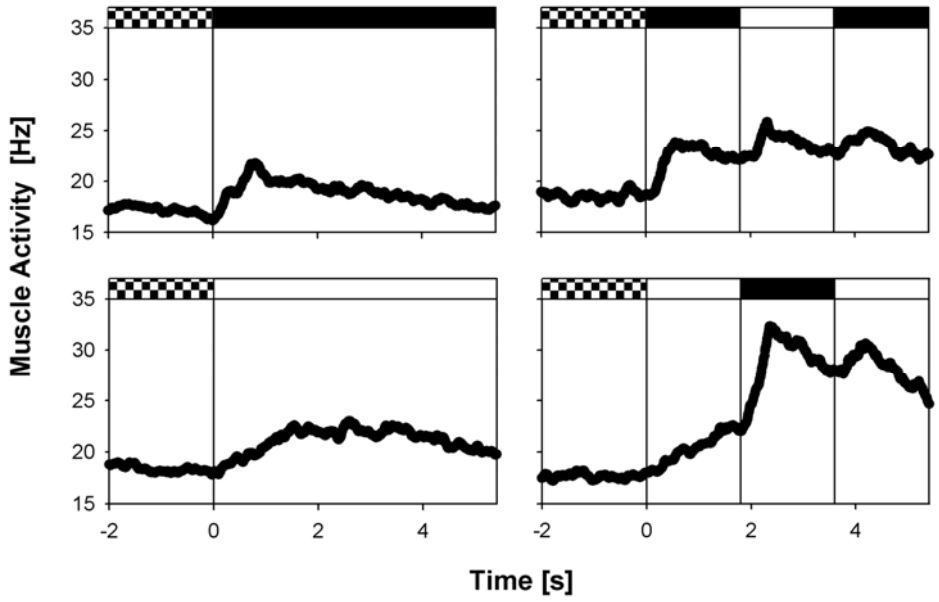


**Fig. 1.** The mean eye muscle activity of 7 spiders with 5 presentations each (35 presentations; bin size 0.2 s) for different stimuli as a function of time. The first vertical lines refer to the change from the checkerboard pattern to either a bright or a dark rectangle and subsequent lines refer to a change from bright to dark or *vice versa,* as indicated at the top of the diagrams.

**Table 1.** Mean changes of eye muscle activity upon stimulus onset ± standard error of the mean (s.e.m.). The significance of the activity increases was tested using the Wilcoxon signed-rank test (N = 7, one-sided value). Stimulus onset is the change from the checkerboard pattern (cb) to a bright or dark rectangle, or to a counter-phase flicker. The rectangle either remains bright or dark (step stimulus) or flickers at a given frequency.

| **Stimulus** | **Mean ± s.e.m [Hz]** | **p-value** |
|---|---|---|
| cb to bright (step stimulus) | 3.6 ± 1.4 | 0.0391 |
| cb to dark (step stimulus) | 3.0 ± 0.8 | 0.0078 |
| cb to bright (0.278 cps) | 9.3 ± 1.5 | 0.0078 |
| cb to bright (0.554 cps) | 8.9 ± 1.8 | 0.0078 |
| cb to bright (1.11 cps) | 7.5 ± 1.4 | 0.0078 |
| cb to dark (0.278 cps) | 7.1 ± 2.7 | 0.0234 |
| cb to counter-phase (0.554 cps) | 4.4 ± 1.1 | 0.0156 |

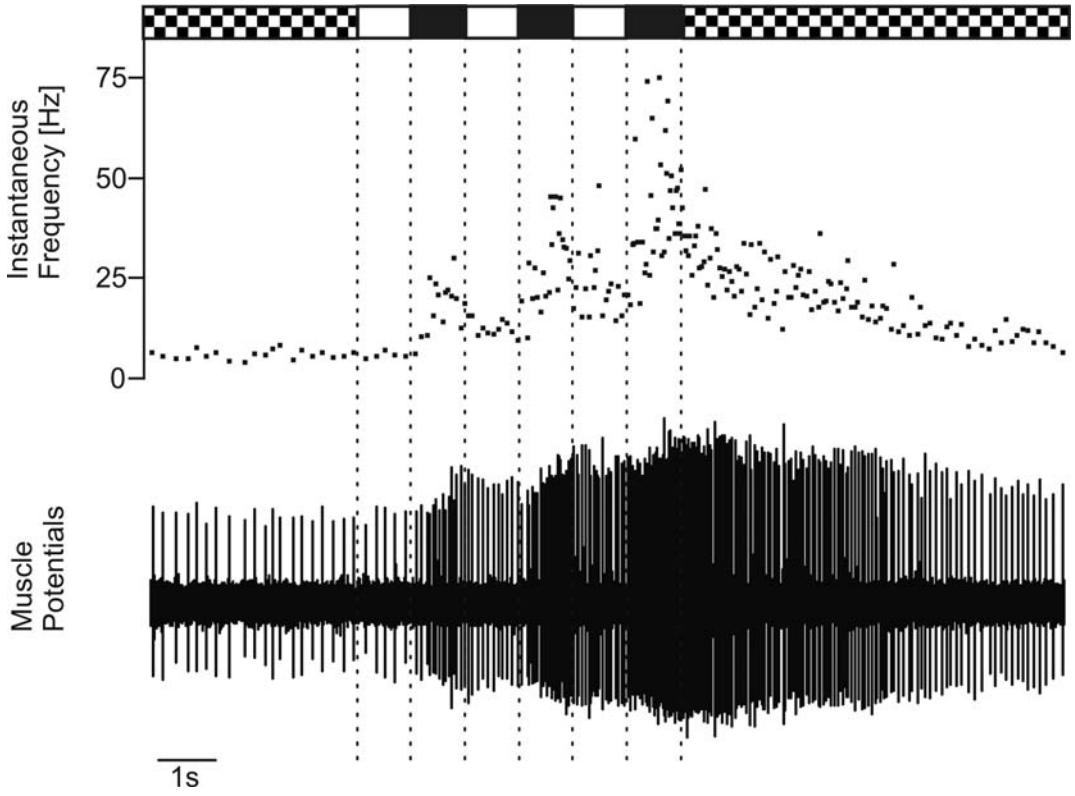


**Fig. 2.** An example of a single stimulus presentation (0.554 cps). The first vertical line indicates the change from the checkerboard pattern to a bright rectangle and subsequent lines indicate a change from bright to dark and *vice versa*. We show a response of the spider that had the lowest resting frequency, because here the changes in eye muscle activity are most distinct. The spider did predominantly respond to a decrease in intensity.
Our experimental setup is not suited for the measurement of the potentials' absolute amplitudes.

**Behavioural cut-off frequency**

The mean frequency changes for the 12 spiders after the onset of the flicker are shown together with the standard error of the mean in Fig. 3. A flicker frequency of 0.554 cps elicited a mean increase of the spiders' eye muscle activity of 9.5 Hz. The response of the spiders falls quickly for higher frequencies and the maximal frequency that elicited a significant increase in our experiments was 4.28 cps ($p = 0.0386$, one-sided value). None of the higher frequencies, and all of the lower frequencies, elicited a significant response (see Table 2). This suggests that the behavioural temporal cut-off frequency lies somewhere between 4.3 and 8.6 cps for the stimuli used.

The temporal patterns of the eye muscle activity for three different flicker frequencies are shown in Fig. 4 (mean of all 72 presentations). The first vertical line indicates the change from the checkerboard pattern to a white rectangle. All subsequent lines indicate a change from bright to dark rectangles and *vice versa*. The response pattern recorded for the slowest flicker frequency (0.554 cps) reveals a rhythmic increase in the muscle activity (Fig. 4A). In Fig. 4B, showing the pattern for the highest frequency that elicited a significant response (4.28 cps), a slight increase upon flicker onset can be seen, with an overall frequency that does not reach the pre-stimulus value in the following three seconds. No distinct peak upon stimulus onset is observed for 8.6 cps, i.e., the lowest frequency that did not elicit a significant response (Fig. 4C).

**Table 2.** Mean changes of the eye muscle activity upon stimulus onset ± standard error of the mean (s.e.m.) for different flicker frequencies. The significance of the activity increases was tested using the Wilcoxon signed-rank test (N = 12). The p-values are one-sided values for frequencies smaller than 8.6 cps (the first negative mean); subsequently we give the two-sided values (in italic).

| **Flicker Frequency [cps]** | **Mean ± s.e.m [Hz]** | **p-value** |
|---|---|---|
| 0.554 | 9.5 ± 1.6 | 0.0002 |
| 1.11 | 8.7 ± 1.2 | 0.0002 |
| 2.21 | 5.1 ± 1.2 | 0.0002 |
| 4.28 | 1.1 ± 0.6 | 0.0386 |
| 8.6 | -0.2 ± 0.3 | *0.5186* |
| 15 | 0.5 ± 0.6 | *0.8501* |
| 30 | 0.3 ± 0.4 | *0.6221* |
| 60 | -0.1 ± 0.2 | *0.9097* |

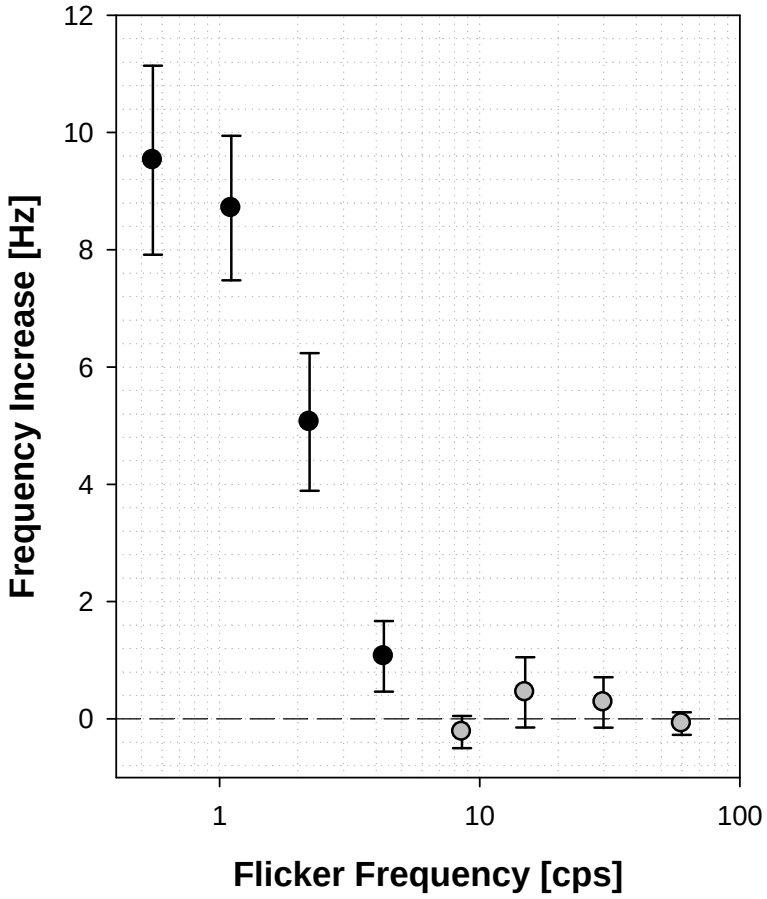


**Fig. 3.** The mean activity increase (± s.e.m.) in the first three seconds after flicker onset compared to the preceding three seconds as a function of flicker frequency for 12 spiders with 6 presentations each. Filled circles give the mean of significant responses; grey circles give the mean of not significant activity changes.

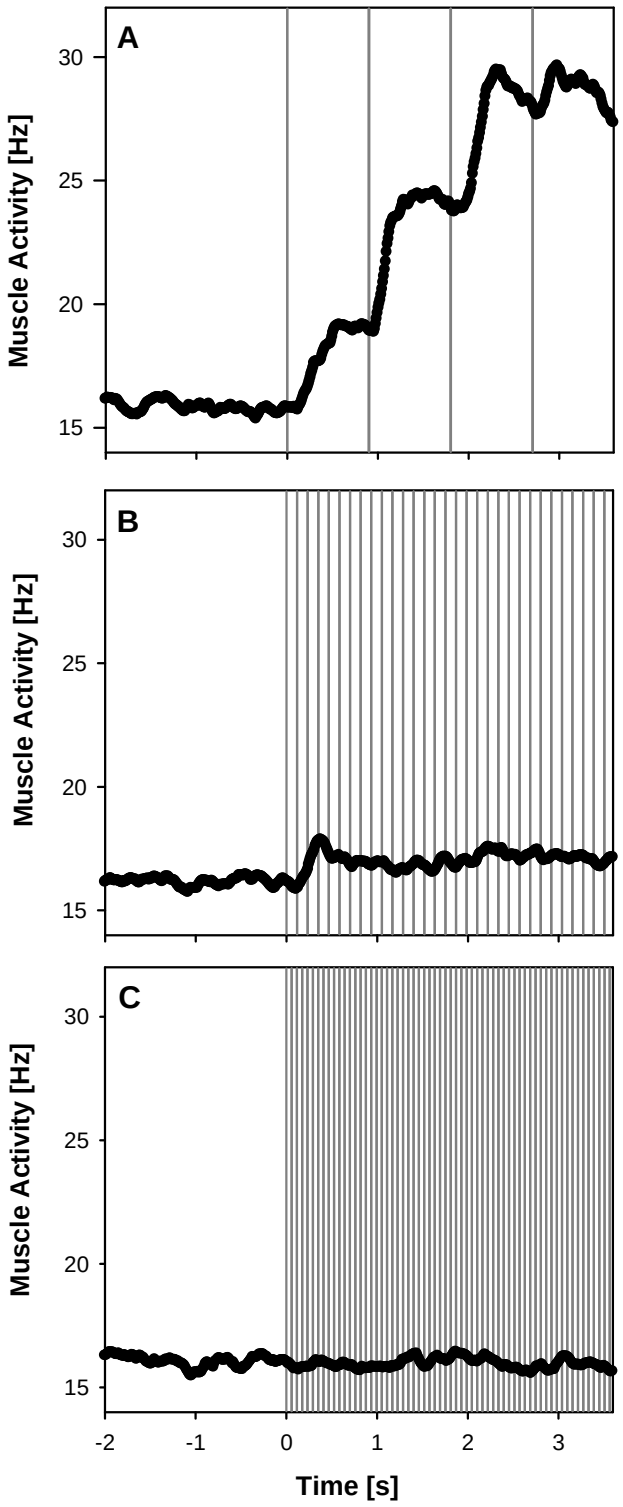


**Fig. 4.** The mean eye muscle activity (72 stimulus presentations, 12 spiders, bin size: 0.2 s) as response to A) the slowest flicker of the second series (0.554 cps), B) the highest frequency that elicited a significant increase (4.28 cps) and C) the lowest frequency that did not elicit a significant increase (8.6 cps).

# DISCUSSION

Our data clearly confirm the hypothesis that *Cupiennius* responds to pure temporal intensity modulations and that no directed motion is required to elicit an increase in eye muscle activity. The net increases in activity of about 9 Hz measured for slow flicker frequencies was higher than the increase determined in previous studies for moving stripes (Neuhofer et al., 2009; Fenk and Schmid, 2010; Orlando and Schmid, 2011). The spiders respond reliably, and for sufficiently low flicker frequencies the averaged activity exhibits a rhythmic increase. The slope of the averaged increase was found to be steeper for an intensity decrease than for an intensity increase. This might be compared to the results of a previous study where we could show that the animals are able to quickly approach visual targets on a computer screen (Fenk et al., 2010b). We found that the spiders were able to follow dark targets on a bright background as well as bright targets on a dark background; however the attack rate was significantly higher for dark targets. Similar findings are reported for the jumping spider *Menemerus bivittatus*, which was also shown to prefer dark targets on a bright background (Tiedemann, 1993).

Due to the limited brightness of CRT monitors, the present setup does not allow the determination of the spiders' maximum temporal cut-off frequency. The illuminance at the animals' position was 8 lx for the screen showing the dark rectangle and 38 lx showing the white rectangle. The lower value is very close to the light intensity that elicited a half-maximum response in ERG measurements (Barth et al., 1993) and is more than 4 log-units above the spiders' threshold. The light intensity at which spiders leave their retreat at dusk is about 20 lx (Barth and Seyfarth, 1979), which is in the same order of magnitude as the intensity of the presented stimuli. Our measurements might thus give the order of magnitude of the behavioural cut-off frequency at maximum light intensities encountered by *Cupiennius salei* during its active period in the natural environment. Pirhofer-Walzl et al. (2007) determined the temporal properties of the photoreceptors using intracellular recordings. In the dark-adapted PME, the time to peak and the integration time were found to be 142 ms and 138 ms respectively, and for light-adapted PME 87 ms and 79 ms respectively (Pirhofer-Walzl et al., 2007). The presentation time of half a cycle for a frequency of 6 cps (the frequency midway between 4 and 8 cps) is 83 ms. The image presentation time, i.e., the presentation time of one bright or dark rectangle, thus matches well the magnitude of the integration time of light-adapted PME photoreceptors.

The eye muscle activity increase elicited by flicker is probably a very direct measure of the behavioural cut-off frequency and might thus reflect the highest frequencies that can be detected by the animals. Here no directional information has to be treated, as it is the case in studies based on optomotor response or prey capture (e.g. Autrum and Stoecker, 1950; Autrum, 1952; Haldin et al., 2009), nor are the animals required to learn, as it is the case in studies based on discrimination tasks (e.g. Srinivasan and Lehrer, 1984a; Srinivasan and Lehrer, 1984b; Railton et al., 2009). The performance of animals might differ considerably according to the behavioural tasks investigated. Autrum and Stöcker showed that optomotor response in honeybees persists up to 200 Hz (Autrum and Stoecker, 1950). In discrimination tasks, however, bees seem to be almost unable to use monochromatic temporal intensity modulations (Lehrer et al., 1993).

It might be interesting to compare *Cupiennius salei* to the toad, a well studied vertebrate with similar lifestyle (see also Pirhofer-Walzl et al., 2007). Both animals are nocturnal predators that remain motionless waiting for prey to pass by. The neural images in the toads' eyes and the spiders' motionless secondary eyes are assumed to adapt and only moving objects would pop up on the animals' retinae (Ewert and Borchers, 1974; Land and Barth, 1992). Toad rod photoreceptor cells have integration times of 1.0-1.3 s at 25°C, and substantially longer integration times at lower temperatures (Haldin et al., 2009) and are thus roughly ten times slower than photoreceptors in *Cupiennius*. A good match was found between the integration time of the toad rod photoreceptor cells at different temperatures and the "exposure time" of a dummy necessary to elicit prey capture (Haldin et al., 2009).

*Cupiennius salei*'s visual system seems to be tuned to the detection of small, slowly moving objects under poor light conditions. The orientation-dependent spatial cut-off frequencies (Fenk and Schmid, 2010) and the different interreceptor angles (Land and Barth, 1992) suggest that object detection and localization should be best in the spiders' frontal plane. The photoreceptor cells subtend smaller angles along the tapetal rows parallel to the spiders' frontal plane than normal to it. The elongated photoreceptor cells might permit to increase photon capture, and thus signal-to-noise ratio in the spiders' dim environment, while maintaining relatively precise spatial information about an objects position in the spider's principal plane of action. The behavioural temporal cut-off frequency of a few cycles per second and the spatial cut-off frequency of 0.5 cycles per degree suggest a predominance of the spatial domain in the trade-off between temporal and spatial resolution compared to other arthropods. Relatively large integration times and relatively good spatial acuity is what one would predict for sedentary animals

interested in small, slowly moving objects (Warrant, 1999). This perfectly meets *Cupiennius salei*'s lifestyle as a typical sit-and-wait hunter that also uses visual cues during prey capture.

## ACKNOWLEDGMENTS

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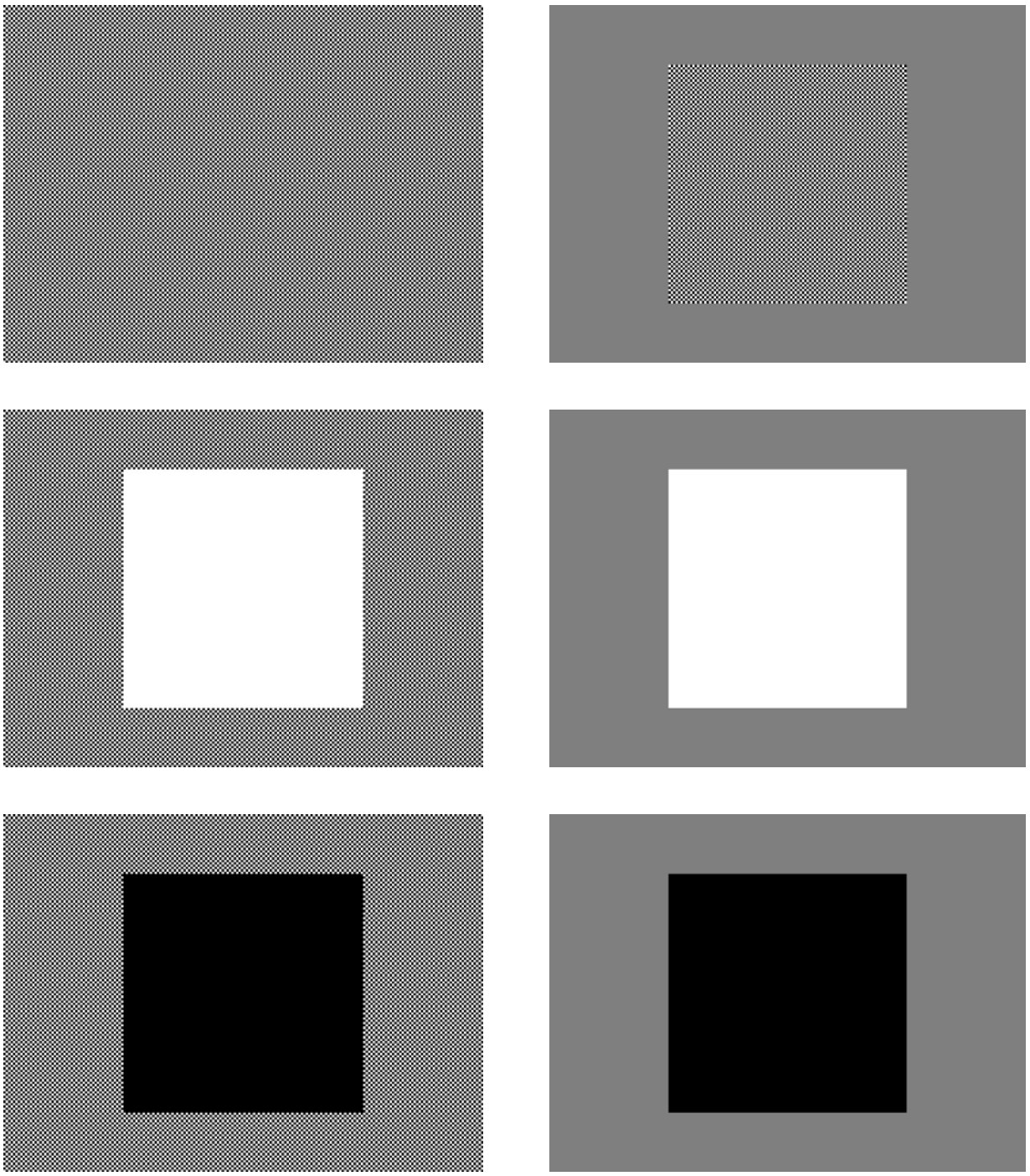

**Suppl. 1.** Screen shots of the stimuli presented in our experiments. The left column shows the stimulus for the first experimental series (slow flicker and step stimuli), the right column the stimuli used in the second series (determination of the behavioural cut-off).

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**Short Communication**

# Vision as a third sensory modality to elicit attack behavior in a nocturnal spider

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## ABSTRACT

*Cupiennius salei* (Ctenidae) has been extensively studied for many years and is probably the only spider that presently can be considered a model organism for neuro-ethology. The night-active spiders have been shown to predominantly rely on their excellent mechano-sensory systems for courtship and prey capture, whereas vision was assumed to play a minor role, if any, in these behavioral contexts. Using slowly moving discs presented on a computer screen it could be shown for the first time that visual stimuli alone can elicit attack behavior (abrupt approaching reactions) in these spiders as well. These observations suggest that visual information could be used by the spiders to elicit and guide predatory behavior. Attack behavior in *Cupiennius salei* can thus be triggered independently by three sensory modalities—substrate vibrations, airflow stimuli, and visual cues—and offers an interesting model system to study the interactions of multimodal sensory channels in complex behavior.

**Electronic supplementary material**
The online version of this article (doi:10.1007/s00359-010-0575-8) contains supplementary material, which is available to authorized users.

**Keywords** Multi-sensory – Spiders – Vision – Attack behavior – Computer screen

# INTRODUCTION

The ctenid spider *Cupiennius salei* is certainly one of the most thoroughly studied species among arachnids and is in particular well known for its excellent mechano-sensory systems. The strictly night-active spiders retreat preferably on monoctyledons during the day time (Barth et al. *1988*) and begin to hunt and search for mates at dusk (Barth and Seyfarth *1979*; Schmitt et al. *1990*).

Pre-copulatory behavior in *Cupiennius* is mediated by pheromones and vibrations and no evidence for visual signaling has been found (Barth and Seyfarth *1979*; Barth *1993*).

It is a well-established fact that both substrate vibrations (Hergenröder and Barth *1983*) and airflow stimuli (Melchers *1963*; Hergenröder and Barth *1983*; Barth et al. *1995*) alone can elicit prey capture in *Cupiennius*. The spiders are very well able to catch prey with blinded eyes, and it has therefore been generally assumed that vision plays only a very minor role, if any, in prey capture.

But the visual system too is very well developed: Land and Barth (*1992*) investigated the optics of the eye and showed that the image of the lens contains much more spatial detail than the photoreceptor array can sample and that spatial resolution is thus limited by the inter-receptor angles. The smallest angles are in the order of 1° and were found in the posterior median and posterior lateral eyes along tapetal rows. The astonishing low F-numbers (Land and Barth *1992*) suggested high light sensitivity, which was indeed confirmed by an absolute corneal illuminance threshold that was found to be clearly below 0.01 lx (Barth et al. *1993*). Likewise, the visual centers in the brain (Strausfeld and Barth *1993*; Strausfeld et al. *1993*) indicate the relevance of the visual sense in at least some behavioral contexts. In behavioral studies *Cupiennius salei* was shown to be able to distinguish vertical from sloped objects (Schmid *1998*) and to switch the mode of locomotion when the light is turned off (Schmid *1997*). The spiders respond to moving objects in the visual field of the secondary eyes with an increase in the activity of the muscles that move the retinae of the principal eyes (Neuhofer et al. *2009*). A significant activity increase in response to moving gratings could be shown for bar widths down to 1° visual angle (Fenk and Schmid *in press*).

These findings lead to the assumption that the eyes could also be used in more demanding behavioral contexts. Thus the aim of this study was to investigate the possible relevance of the visual sense in prey capture behavior.

## MATERIALS AND METHODS

Five- to six-month-old *Cupiennius salei* from our stock in Vienna were used in this study. The spiders were bred and raised in a greenhouse in Vienna at a relative humidity of 70–80% and a temperature of 23–28°C. The spiders were kept separately in glass jars and were fed flies once a week.

During the experiments the spiders remained in their habitual glass jars. The animals were thus shielded from air movements that could have interfered with the experiments and did not have to adjust to a new environment.

In each session four spiders were positioned simultaneously in front of two 22" computer screens (Samsung SyncMaster T220). The distance between the spiders' eyes in their initial position and the screen was 15–20 cm. Styrofoam plates were placed equidistantly between the glass jars so that each spider was given a view of half the screen and the spiders could not see each other. The individual jars were placed on foam material to insulate them mechanically and to reduce vibrations induced by moving spiders that could possibly irritate the other animals.

The spiders have a very pronounced preference to sit in their glass jars with their prosoma pointing downwards. At the beginning of the experiments the glass jars were oriented in such a way that the spiders sat at the most distant side of the jar with their dorsal side and the eyes turned towards the screen.

Three different stimuli were generated using Microsoft PowerPoint. The first one consisted of a black disc that appeared slowly and then moved for 40 s in jerky curves in front of a green background. The pathway of the disc is shown in Fig. 1a. The visual angle of the disc at the distance of the spiders' initial position was in the order of 8.5°. The second stimulus was identical to the first one except that the contrast was inverted, i.e., a green disc was shown in front of a black background. The third stimulus was a control and consisted of a green background and a disc in a slightly different green. This control was chosen to ensure that processor and screen activity, and subsequent fan activity, was comparable to the activity during the presentation of the actual test stimuli. A green rather than a white background was used because the angular properties of the color rendering of the screen were less variable using only one of the three color channels. The spectral composition of the green channel lies well within the receptive spectrum of the spiders' eyes reported by Barth et al. (*1993*). The illumination level was measured to be in the order of 25 lx at the spiders' initial position pointing at the green screen (MT-51, Voltcraft).

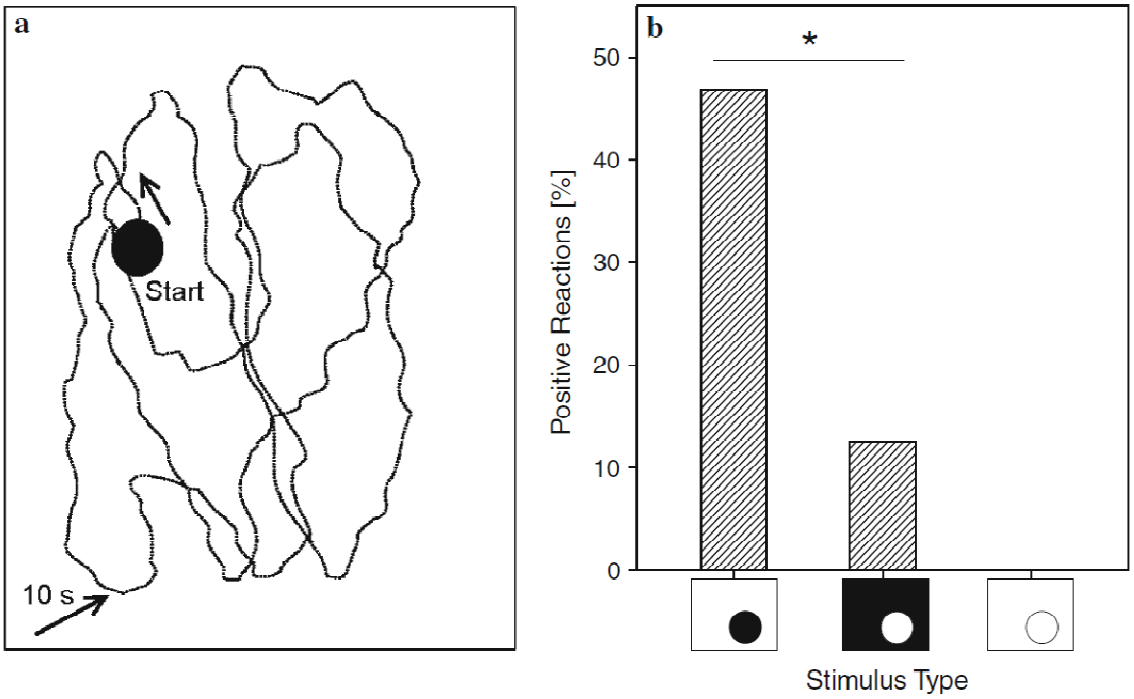


**Fig. 1** **a** The pathway of the moving disc (diameter $d = 2.6$ cm) on half of the screen. The *upper arrow* gives the starting direction; the *lower arrow* indicates the position of the disc 10 s after movement onset. The height of the screen was about 29.5 cm. **b** Percentage of sessions in which one or more positive reactions were recorded ($n = 32$). The spiders responded significantly more often to the black disc on a green background than to the inverted stimulus, that is, a green disc on a black background. ($p = 0.016$, $n = 8$, calculated with the Wilcoxon signed-rank test, two-tailed value). No reactions to the green disc on green background were recorded

The moving discs were presented simultaneously to two spiders while the neighboring spiders were only confronted with the respective background. This stimulus inter-stimulus sequence was repeated three times for each stimulus type before the next type was shown. Then the positions of the spiders were interchanged and the sequence was shown once again. We repeated this procedure with the same spiders but with an inverted order in which the three different stimulus types (black disc, green disc, and control) were presented.

The spiders and the stimuli were filmed simultaneously with two web cameras (Logitech Webcam Pro 9000). We counted a reaction to the stimulus as positive when the spiders abruptly approached the disc on the screen. In *Cupiennius salei* quick approach reactions, like jumps or the abrupt turning of the spiders towards a stimulus source, have been used as indicators of predatory behavior in studies investigating its mechano-sensorical guidance (Hergenröder and Barth *1983*; Barth et al. *1995*). The spiders seldom move without stimulation and in the rare cases they do so the movements are very slow and limited to a small perimeter and can easily be distinguished from the reactions to the presented stimuli. We observed such spontaneous movements only once during the experiments reported here.

## RESULTS

In the first series, seven out of the eight spiders reacted to the black disc moving in front of the green background, and in the second series four spiders out of eight reacted to this stimulus. Pooling the two sessions all spiders responded at least once to the stimulus. In 15 out of 32 presentations there were one or more positive reactions to the dark disc. The inverted stimulus, i.e., the green disc moving in front of a black background, elicited only 4 positive reactions during 32 presentations. For the control stimulus no reactions were recorded. The results are summarized in Fig. 1b.

To test whether the spiders responded significantly more often when the disc was darker than the background than the other way around, we compared for each spider in how many single presentations reactions were observed. Seven out of eight spiders responded more often to the dark disc, one spider responded equally often to the two stimuli, and the difference showed indeed to be significant ($p = 0.016$, $n = 8$, calculated with the Wilcoxon signed-rank test, two-tailed value, using MATLAB R2006a).

The mean time span (± standard error) between the movement onset of the disc and the first reaction of the spiders was 9.6 s ± 1.2 s for the black disc ($n = 15$) and 10.1 s ± 0.8 s for the green disc ($n = 4$).

The spiders react to the stimuli in a rather abrupt way. Many animals initially raise the forelegs before they rapidly approach the target; other animals were observed to approach the targets out of their fright posture and at several occasions the spiders even bumped into the glass walls of their habitual jars. When the spiders reach the opposite side of the jar facing the target with their ventral side they are still able to follow the moving disc performing grasping movements with their forelegs. Fig. 2 shows superposed single frames of the videos taken in this study to illustrate the spiders' reactions and video clips are provided as online material.

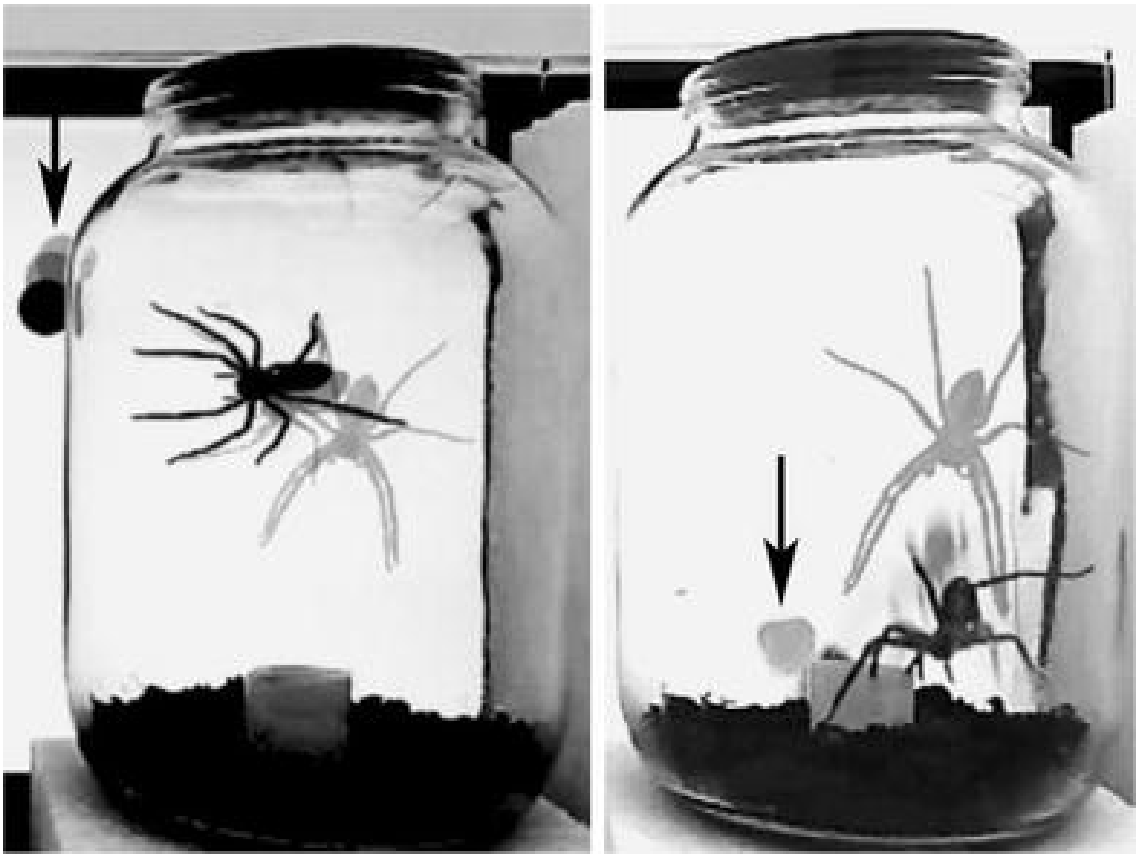

**Fig. 2** Three snapshots taken from the videos recorded during the experiments were superimposed on each figure to illustrate the spiders' reactions. The *arrows* indicate the position of the stimulus on the screen just before the spiders' reactions. The spider is depicted in *light gray* in its initial position and in *black* in its final position. The turning reaction on the left side was completed in 0.80 ± 0.08 s, the leap on the right side in 0.46 ± 0.08 s (where the uncertainty is due to the frame rate of 12.5 frames per second)

## DISCUSSION

We could show that visual stimulation alone can elicit attack behavior (i.e., quick approaching reactions) in *Cupiennius salei* and our results suggest that the spiders could use visual information for hunting and aggressive defense behavior.

Dark targets on bright backgrounds seem to be much more efficient stimuli than bright targets on dark backgrounds for *Cupiennius salei*. This might be compared to the findings of Tiedemann (*1993*) who studied brightness discrimination in the salticid *Menemerus bivittatus* by presenting circular prey stimuli with varying gray values in front of a white, gray, and a black background. The response rate increased faster with increasing contrast when the stimulus is darker than the background than when the stimulus is brighter than the background. Although prey detection mechanisms are probably very different from those known in vertebrates, it would make sense for a sit and wait hunter to have detectors similar to "bug perceivers" in frogs

(Lettvin et al. *1959*) that respond to convex dark objects entering their receptive field and moving within this field.

Most spiders started to react around 10 s after movement onset, and this corresponds to a time interval in which the disc was moving for the first time into the lower part of the screen. A rather obvious reason for that might be that it is a lot easier to catch prey during a downward movement rather than to jump or run upwards, opposed to gravity. This is especially true for spiders as large and heavy as *Cupiennius salei*, and it might also explain why the spiders are nearly always positioned with their prosoma pointing downwards (see also Nakata and Zschokke *2010*).

The distance of the screen could theoretically be estimated by the spiders using non-pictorial cues provided via motion parallax, accommodation, and binocular disparity; for an animal that uses these cues the monitor and the scene shown on the monitor would appear at the same depth plane (Zeil *2000*). It is highly unlikely that *Cupiennius* uses cues from binocular disparity or accommodation since the visual fields of the individual eye pairs have no important binocular overlap (Land and Barth *1992*) and no accommodation mechanisms are known in spider eyes. No motion parallax is induced by the spiders remaining stationary before they jump at once towards the target. It is therefore hard to imagine that the spiders could have estimated the distance to the screen and there were probably no cues telling the spiders that the discs and the screen are in the same plane of depth. We thus assume that the animals jumped towards the moving targets without knowing their real size. In natural situations, however, several additional visual cues and also cues involving other sensory modalities are available that could in principle be used to estimate the distance and size of a moving object.

There are several spiders already known to use their visual sense when capturing prey. Salticidae, the spiders with by far the highest spatial resolution are known to hunt primarily with their visual sense and they are reported to visually discriminate mates and prey (Homann *1928*). *Evarcha culicivora* can even identify blood-fed female mosquitoes via their visual sense (Jackson et al. *2005*) just to cite one stunning example for the visual capacities of jumping spiders. Ctenid spiders have a visual acuity that is up to two orders of magnitude worse than that of salticids, and the visual cues that can be used by *Cupiennius* in prey capture behavior are certainly less complex than in most salticids.

Spiders from several families are known to be able to use multiple cues in prey capture behavior. Forster (*1982*) reports that *Trite planiceps*—a salticid known to use visual cues in prey capture (Forster *1979*)—is also able to catch flies in complete darkness or with covered eyes, probably being guided by the airflow emitted by the flies at distances smaller than 1–2 cm. According to Taylor et al. (*1998*) all 17 subfamilies of the salticids investigated were able to

catch prey in the absence of visual cues. However, it seems difficult to judge from the reported experiments whether airflow stimuli, substrate vibration, direct physical contact, or a mixture of these cues drive the actual capturing. The ogre-faced spider *Deinopis spinosus* uses primarily airflow stimuli for backward strikes targeting flying prey and visual cues for forward strikes to catch walking prey in a very stereotyped manor (Coddington and Sobrevila *1987*). The patch residence time in the lycosid *Schizocosa ocreata* is strongly influenced by visual and chemical cues (Persons and Uetz *1996a*,*1996b*) and the species is also reported to attack video playbacks of crickets (Persons and Uetz *1997*). On the other hand, Lizotte and Rovner (*1988*) report nocturnal lycosids to rely more strongly on their mechano-sensory system than on their visual system when hunting fireflies. Lycosids are furthermore known to use multisensory cues during courtship (Uetz and Roberts *2002*).

To our knowledge *Cupiennius salei* is the first spider species for which it could explicitly be shown that attack behavior involves three separate sensory modalities and that all three modalities—vibrations on the substrate perceived using slit sensilla, airflow stimuli perceived using trichobotria, and visual cues perceived using the eyes—are each alone sufficient to elicit it. This raises the quite intriguing question where and how the sensory information is processed. Given the amount of physiological and neurobiological data available for *Cupiennius salei*, this spider would certainly be an interesting model to study the interaction of different sensory channels in the structuring of complex behavioral patterns.

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# Pupil Size in Spider Eyes Is Linked to Post-Ecdysal Lens Growth

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## ABSTRACT

In this study we describe a distinctive pigment ring that appears in spider eyes after ecdysis and successively decreases in size in the days thereafter. Although pigment stops in spider eyes are well known, size variability is, to our knowledge, reported here for the first time. Representative species from three families (Ctenidae, Sparassidae and Lycosidae) are investigated and, for one of these species (*Cupiennius salei*, Ctenidae), the progressive increase in pupil diameter is monitored. In this species the pupil occupies only a fourth of the total projected lens surface after ecdysis and reaches its final size after approximately ten days. MicroCT images suggest that the decrease of the pigment ring is linked to the growth of the corneal lens after ecdysis. The pigment rings might improve vision in the immature eye by shielding light rays that would otherwise enter the eye via peripheral regions of the cornea, beside the growing crystalline lens.

# INTRODUCTION

Most spiders have eight simple eyes that can be divided into two different classes according to structural and functional differences. The anterior median eyes (AM eyes) are referred to as principal eyes. This eye pair points forward and the retinae can usually be moved by a varying number of eye muscles. AM eyes are everted eyes with the light absorbing segment of the photoreceptors turned towards the incident light. The other eye pairs – the posterior-median (PM), the posterior lateral (PL) and anterior-lateral (AL) eyes – are referred to as secondary eyes and can cover various fields of view. Their retinae cannot be moved and the photoreceptor segment bearing the microvilli is turned away from the incident light. The secondary eyes of most nocturnal spiders are equipped with a light-reflecting tapetum. For reviews dealing with spider eyes see e.g. [1], [2].

Pigment rings restrict the aperture in spider eyes. This iris, consisting of pigment cells situated between the rear surface of the cornea and the glassbody, was recognized in the early 19$^{th}$ century [3]. We will refer to the opening left by the pigment ring as “pupil”. The diameter of the pupil in different spider species has been determined in numerous studies and was, implicitly or explicitly, assumed to be constant (e.g. [4], [5], [6], [7], [8], [9]). The extent or absence of the pigment stop in jumping spiders has been shown to be linked to habitat illumination: species observed in shaded forest habitats lack significant pigment stops whereas species living in sunny habitats show extensive pigment rings that can reduce the light flux into the eye by 50% [7].

We first observed variable pigment rings in *Cupiennius salei* (Ctenidae). The pupil's initial size and its successive post-ecdysal growth were determined for three groups of different ages. We hypothesized this process to have a causal connection to the maturation of the eyes after ecdysis and therefore expected post-ecdysal pigment-rings to be found in other spider families as well.

# MATERIALS AND METHODS

## Post-ecdysal pupil size and its subsequent increase in *Cupiennius salei*

The nocturnal hunting spider *Cupiennius salei* is common in Central America. The animals used in this study were bred separately in glass jars (5 liter) in a greenhouse at 12 h:12 h day:night cycle and were fed *Calliphora sp*. once a week. The temperature (15–28°C) and relative humidity (70–80%) resembled the natural conditions found in *C. salei*'s habitat.

*Cupiennius salei* molts 11 times before it reaches adulthood [10], with the first instar outside the eggsac having a body size of about 2 mm and adults reaching a body length of up to 50 mm with a leg span of more than 120 mm. Under laboratory conditions the development is usually completed after one year, and the total life span is in the order of two years. Nine juvenile *Cupiennius salei* were selected for our experiments, forming three classes of spiders of different ages (five, seven and nine months respectively).

In order to determine the moment of ecdysis for each spider, a web camera was positioned in front of the glass jars containing the individual animals and was set to take a picture every five minutes. For the functioning of the camera, the light intensity at night time had to be slightly increased. The characteristic molting positions of the spiders, as described by Melchers [10], could easily be detected in the photos: *Cupiennius* prepares for ecdysis by attaching itself horizontally to a thread with its dorsal side pointing downwards. After the cuticle of the carapax has opened, the pedipalps, the legs, and finally the opisthosoma are extracted from the integument. Once ecdysis is completed, spiders perform characteristic leg movements that prevent the cuticle in the joint region from hardening [10].

After ecdysis the size of the pupil was measured. The animals were anaesthetized with $CO_2$, or cooled down, and subsequently tethered onto a wooden spherical cap. The cap's base had a diameter of 105 mm and the spiders' legs could be attached to the cap with a piece of Parafilm without being bent. A hole in the Parafilm strip left the prosoma and opisthosoma free (see also [11]). The cap was connected to a magnetic stand by means of a ball bearing. By rotating the cap each eye could then be positioned in the horizontal plane under a reflected-light microscope to be photographed (Nikon DS-U1, Adaptors: Nikon Digital Sight DS-U1 and Camera Adaptor CMA-D2, Tokyo, Japan).

The eyes of each spider (left and right AM; left and right PM) were photographed once before ecdysis, and then daily during the first week and every second day during the second week. The lens and pupil diameters were measured using the program Lucia General 5.10 (Laboratory Imaging, Prague, Czech Republic). Both the lens and the pupil have a rather circular shape and the diameters were determined via an approximated circle calculated from a varying number of points that were placed by hand on the outer edge of the pupil and the lens on the images.

The uncertainty of the pupil diameters was estimated by the standard deviation calculated for the diameter of a given lens measured on the different photographs. It was found to be in the order of 5–10 μm, corresponding to roughly 1% of the lens diameters.

## Variable pupil size in other spider families

Two other spider species were examined for variable pigment rings: *Lycosa tarentula* (Lycosidae), belonging, as well as *Cupiennius*, to the superfamily of Lycosoidea, and *Heteropoda venatoria* (Sparassidae) belonging to the group Dionycha [12]. Five 4-month-old *H. venatoria* and one *L. tarentula*, that was just before its penultimate ecdysis, were kept in our laboratory under a 12 h:12 h day:night cycle. The *Heteropoda* spiderlings were placed in small glass jars (0.4 liter) and the *Lycosa* in a plastic terrarium (11 liter). The spiders were fed either flies (*Calliphora* sp.) or crickets (*Acheta domesticus*) once a week.

Since the objective here was only to document the variability of the pupil size, we did not determine the precise moment of ecdysis, but instead checked for exuviae on a daily basis.

One day after ecdysis and once again several days later the spiders were tethered and photographed as described above for *Cupiennius salei*.

## The function of the pigment stop

We tested the hypothesis that the pigment ring is linked to the growth of the corneal lens after ecdysis using X-ray microtomography (microCT) imaging of the cephalothoraxes of two *Cupiennius salei* – one spider was fixed 9 hours after ecdysis and the other 9 days after ecdysis. Both spiders were photographed, as described above, before they were prepared for the scans. The preparation followed the methodology proposed by Metscher [13], [14]: The samples were fixed in Bouin's solution which was washed out two days later with 70% ethanol. After dehydration in 100% ethanol the specimens were stained overnight with iodine (1% $I_2$ in 100% ethanol). For the scans the samples were placed in small polypropylene tubes filled with 100% ethanol.

Both spiders were scanned with 66 kV and 133 µA at a 4.3 fold optical magnification (Xradia MicroXCT, Pleasanton, California), resulting in a projection image pixel size of 2.5 µm for the spider fixed 9 days after ecdysis and 2.0 µm for the other, slightly smaller, spider. Images were reconstructed with 2×2 pixel binning to reduce noise, resulting in final voxel sizes of 5.0 µm and 4.0 µm. Using the Xradia viewer software (TXM 3DViewer), virtual sections at any orientation through the reconstructed eyes could be viewed.

# RESULTS

## Post-ecdysal pupil size and its subsequent increase in *Cupiennius salei*

The enlarged pigment rings were observed in both the principal and secondary eyes and at all three developmental stages examined (Fig. 1A, B). The mean diameters of the lenses of the AM and PM eyes for the three groups before and after ecdysis are given together with the initial pupil size estimated from extrapolating linear regressions calculated for a time $t$<130 hours after ecdysis in Table 1, 2. The initial pupil-to-lens ratio was found to be in the order of 0.5, i.e. only a fourth of the total projected lens surface is free from the pigment shield shortly after ecdysis.

In the following days the pupil diameter $d$ increased and the pupil-to-lens ratio converged towards a ratio of approximately 0.9. The growths of the PM and AM eye pupils of a 7-month-old spider with time $t$ after molting are shown in Fig. 1A together with a sigmoidal fit ($r^2$>0.999) following $d = d_f - a/(1+\exp((t-t_0)/b))$. In both eye pairs 95% of the asymptotic values $d_f$ were reached after roughly 210 h.

The increase of the pupil diameter was found to be rather similar for the 7- and 9-month-old spiders, whereas the 5-month-old spiders seemed to have a slightly larger pupil-to-lens ratio after ecdysis which increased faster in size in the following days as compared to the older spiders (Fig. 1B).

**Table 1. Lens and pupil diameters of the PM eyes.**

| | *d* of lens before ecdysis | *d* of lens after ecdysis | *d* of initial pupil |
|---|---|---|---|
| 5-months (N=4) | 645 | 763 | 417 |
| 7-months (N=3) | 797 | 933 | 462 |
| 9-months (N=2) | 906 | 1009 | 527 |

The means of the diameter $d$ (in μm) of the PM eye lenses for the three different age groups are given for N spiders before ecdysis and after ecdysis. The last column gives the values for $t = 0$ calculated from a linear regression for the first 130 hours after ecdysis as an estimate of the pupils' initial diameter.

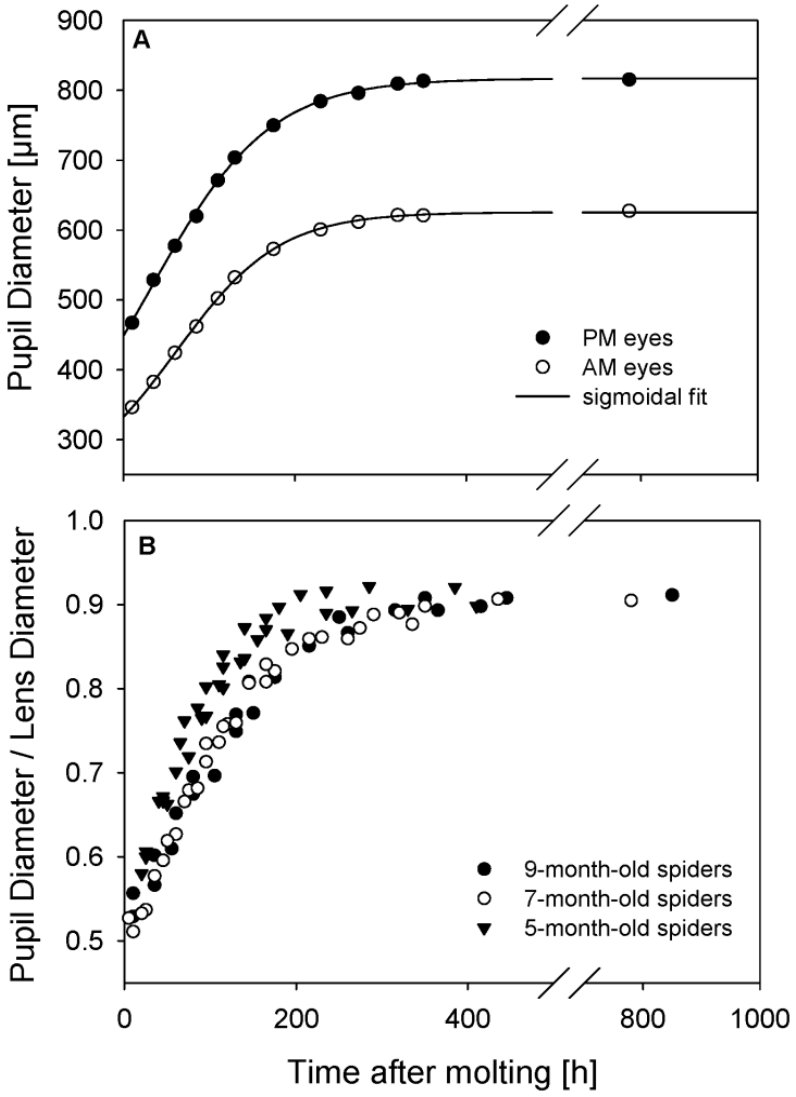


**Figure 1.** (A) The mean pupil diameters of the PM and AM eyes for a 7-month-old spider as a function of the time after ecdysis. The data were fitted using $d = d_f - a/(1+\exp((t-t_0)/b))$, where $d$ is the pupil diameter and $d_f$ is its asymptotic value for large times $t$. PM eyes ($r^2 = 0.9993$): $d_f = 817$ µm, $a = 600$ µm, $t_0 = 32$ h, $b = 69$ h. AM eyes ($r^2 = 0.9997$): $d_f = 626$ µm, $a = 400$ µm, $t_0 = 61$ h, $b = 60$ h. (B) The pupil-to-lens ratios of the PM eyes of *Cupiennius salei*, for three different age groups as a function of the time after ecdysis.

**Table 2. Lens and pupil diameters of the AM eyes.**

| | *d* of lens before ecdysis | *d* of lens after ecdysis | *d* of initial pupil |
|---|---|---|---|
| 5-months (N=4) | 445 | 559 | 289 |
| 7-months (N=3) | 598 | 716 | 327 |
| 9-months (N=2) | 670 | 775 | 364 |

The means of the diameter *d* (in µm) of the AM eye lenses for the three different age groups are given for N spiders before ecdysis and after ecdysis. The last column gives the values for $t = 0$ calculated from a linear regression for the first 130 hours after ecdysis as an estimate of the pupils' initial diameter.

## Variable pupil size in other spider families

To test if a variable pupil size can be found in other spider species, we investigated *Lycosa tarentula* and *Heteropoda venatoria*. Both species indeed showed large pigment rings after ecdysis that disappeared almost entirely in the days thereafter. Pictures of *C. salei*, *L. tarentula* and *H. venatoria* one day after ecdysis are shown in Fig. 2.

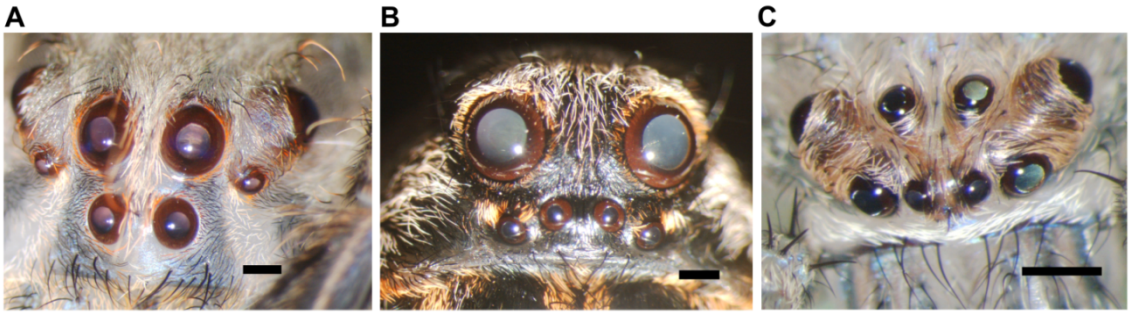


**Figure 2.** Portraits of *Cupiennius salei* (A), *Lycosa tarentula* (B) and *Heteropoda venatoria* (C) the day after ecdysis. In all three species the iris formed by the pigment ring is clearly visible and was observed to disappear in the days thereafter. Scale bars: 500 µm.

## The function of the pigment stop

Slices through the reconstructed eyes of the spider scanned shortly after ecdysis show small lenses attached to the most central part of the corneal cap. The scan of the second spider, which was fixed 9 days after ecdysis, reveals a lens that has clearly increased in size compared to the cornea, and that now fills out more than the corneal cap.

A comparison of the *in vivo* micrographs with virtual slices in the plane of the PM eyes indicates a good matching of the part of the cornea covered by the lens and the pupil diameter (Fig. 3). Both the ratio of the pupil diameter to the lens diameter in the micrograph, and the ratio of the crystalline lens diameter at the corneal surface to the cornea diameter, is in the order of 0.55 in the post-ecdysal eye.

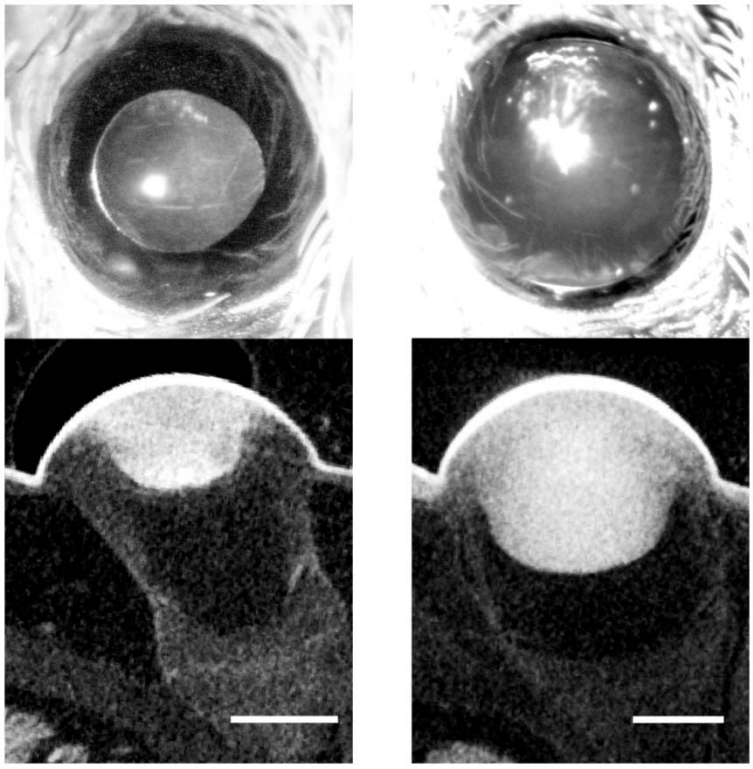

**Figure 3.** The upper part shows *in vivo* micrographs of a PM eye of a spider the day after ecdysis (left panel) and 9 days after ecdysis (right panel), taken just before the preparation of the spiders for the micro CT scans. The lower part shows sections through the reconstructed PM eyes. Scale bars: 200 μm.

## DISCUSSION

A detailed description of the molting of spider eyes is given by Wagner [15]. He recognized that the cuticular cornea and parts of the crystalline lens are formed before ecdysis and that the crystalline lens grows in the days thereafter. Browning reported similar findings for *Tegenaria* in a study that deals primarily with the structure of the cuticle before and after molting [16]. Our observations clearly confirm these descriptions. However, the crystalline lens seems to be initially restricted to the middle of the corneal cup (Fig. 3), which could not be derived from Wagner's and Browning's drawings. We assume that the more peripheral parts of the cornea, which are not covered by the growing lens, are shielded by the pigment rings. Wagner did certainly also observe the pigment rings, since he stated that in *Lycosa* only three quarters of the cornea are transparent. Interestingly, he reported the same for the cornea shed with the exuvia, whereas we found all parts of the eyes in the exuviae to be completely transparent. Moreover, Wagner did not mention any temporal variability of the pupil size and finally concluded that spiders probably have a limited field of view.

Future research on the visual systems of spiders should take the post-ecdysal development of the lens into account. Measurements of the aperture of the eyes are certainly difficult to compare without knowledge of the time since the last molt – at least for some spider species. On the other hand, large pigment rings might allow the identification of freshly molted individuals collected in their natural environment.

In kissing bugs (*Triatoma infestans*) Insausti and Lazzari [17] have specified a similar process, as we describe here for spiders. In *T. infestans* recently emerged adults show an elongated narrow pupil surrounded by pigment cells. The pupil widens during the following twenty days, and the change in pupil size corresponds well to the growth of the corneal lens. The authors suggest that the pigment rings are linked to the development of the ocelli [17]. Ocelli of insects can also be equipped with pupils that change size as a response to light stimuli as has been observed in two locust species [18].

In *Cupiennius salei* the lens in the mature eye produces an image of good quality on the retina as has been shown by Land and Barth using an ophthalmoscope [19]. A significant behavioral reaction of the spiders to moving gratings was measured down to spatial periods of 2° [20], which also indicates that the optical system is well developed in this nocturnal species.

We measured the radii of curvature of the corneal lens and the distance between the lens surfaces and the retina on the microCT sections for a *Cupiennius salei* fixed nine days after ecdysis. Considering these measurements, a homogeneous crystalline lens must be assumed to have a refractive index in the order of 1.67 to achieve enough refractive power to focus parallel rays on the retina. However, such a high refractive index is not common in biological materials and, as argued by Blest and Land, this suggests the existence of a graded refractive index, which could also correct for spherical aberrations [21]. Blest and Land investigated the large PM eyes of *Dinopis* and compared the radii of curvature to the focal length measured in excised lenses. Based on the radii of curvature the authors calculated the focal length as a function of the refractive index and found that a refractive index of 1.65–1.67 would be needed to achieve the focal length actually measured in *Dinopis*. The high apparent refractive index was taken as an indication for an inhomogeneous structure of the lens. This assumption was also corroborated by a less pronounced spherical aberration measured in these eyes than would be expected for a homogeneous lens [21]. If we assume this apparent refractive index for the eye of *Cupiennius salei* fixed nine hours after ecdysis, the calculations suggest that the image of an object at infinity is well focused on the retina for light rays passing through the lens and that the growing lens can already produce a sharp image on the retina.

The pigment rings might attenuate the impairment of vision which would result from peripheral light rays entering the eye beside the lens. In the absence of shielding pigment two

posterior nodal distances must be expected in the immature eye: one for light passing only through the curved cornea, and a shorter one for light passing through the cornea plus the crystalline lens. If light rays were permitted to enter the eye beside the lens via the outer regions of the cornea, a second focal plane would be formed roughly 600 μm behind the retina, and additionally a strong spherical aberration should be expected. This would certainly severely degrade the quality of the image formed by the crystalline lens on the retina. But since the pigment rings restrict the aperture there are no reasons to believe that spatial resolution is much impaired after molting. However, the image on the retina must be expected to be less bright due to the smaller pupil and the accordingly higher F-number.

The total time in which spiders have to deal with immature eyes is by no means negligible and it might therefore be interesting to investigate in behavioral studies to what extent vision is available shortly after ecdysis.

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## AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: LMF AS. Performed the experiments: LMF KH PL. Analyzed the data: LMF KH PL. Contributed reagents/materials/analysis tools: AS. Wrote the paper: LMF. Read and commented on the paper: AS.

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**Fenk, L. M. and Schmid, A.** (2010). The orientation-dependent visual spatial cut-off frequency in a spider. *J. Exp. Biol.* **213**, 3111-3117.

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Data Collection, Data analysis, Model, and Writing of the Manuscript: LMF.

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**Fenk, L. M., Hoinkes, T. and Schmid, A.** (2010). Vision as a third sensory modality to elicit attack behavior in a nocturnal spider. *J. Comp. Physiol. A* **196**, 957-961.

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**Fenk, L. M., Heidlmayr, K., Lindner, P. and Schmid, A.** (2010). Pupil size in spider eyes is linked to post-ecdysal lens growth. *PLoS ONE* **5**, e15838.

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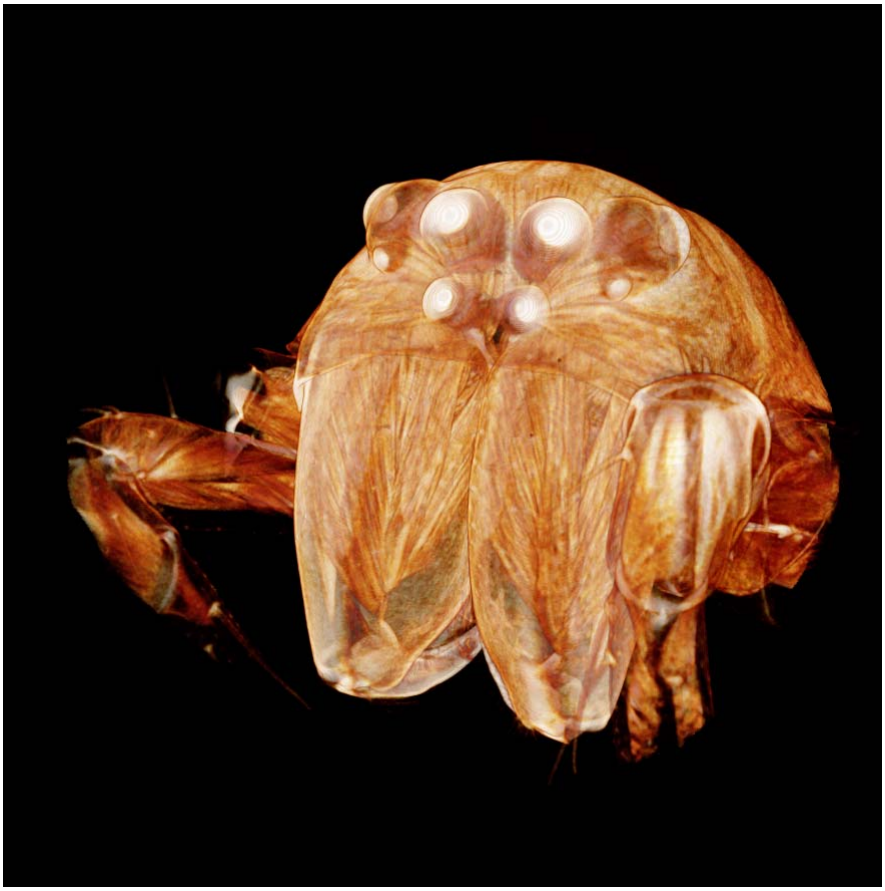

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Fenk LM, Schmid A. 2010. The orientation-dependent visual spatial cut-off frequency in a spider. *J Exp Biol* 213(18):3111-3117.

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## Conference Contributions

(Presentations by first authors)

Fenk LM, Schmid A. 2011. *The behavioral spatial and temporal cut-off frequencies of the visual system in a nocturnal spider*. 13th International Neuroscience Winter Conference, Sölden, Austria. (Poster)

Fenk LM, Schmid A. 2010. *Spider Vision*. ANN (Arthropod Neuro Network) Symposium in Hamburg, Germany. (Talk)

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Fenk LM, Schmid A. 2009. *The orientation dependent visual acuity in a wandering spider*. AVA Animal Visual Ecology Symposium at ECVP 09 in Regensburg, Germany. (Talk)

Fenk LM, Schmid A. 2009. *Spatial acuity of the PM eyes in a hunting spider.* Austrian Neuroscience Association Meeting 2009 in Salzburg, Austria. (Poster)

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