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Titel der Masterarbeit

Colour Vision in *Cupiennius salei* - a Behavioural Approach with Virtual Reality

verfasst von

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For
Nina

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Contents

Abstract

The visual system of the nocturnal hunting spider *Cupiennius salei* (KEYSERLING, 1877) is one of the best investigated of all spiders. This spider has eight eyes in the frontal region of the prosoma, arranged in two rows. Electroretinograms and intracellular recordings show a broad spectral sensitivity from about 300 nm - 650 nm. Furthermore, three types of photoreceptor cells with different spectral peak sensitivities at UV (340 nm), blue (480 nm) and green (520 nm) were found. These findings are also reflected in behavioural experiments, but showing a shift to longer wavelengths. These findings raise the question if *C. salei* uses colour as an additional cue to assess their environment.
On several occasions it has been shown that this species is attracted to square shaped vertical targets. In this study, this behaviour is used to test if *C. salei* can discriminate a target from a background only by its chromatic properties. For this reason homogeneous backgrounds, either blue or green, were used in combination with darker or brighter targets of the opposite colour. The contrast ratio was changed by altering the intensity of both colours. As the environment was otherwise as feature less as possible, the target was providing the only vertical structure and therefore should be preferred by the spider. If colour vision is used in this task, spiders should be able to detect and approach the target in all contrast ratios/settings.

The response was tested in two different experimental set-ups, both using the same technique of projecting the target and the background on a white surface. Experiment I was conducted with free running spiders in a 210 cm x 210 cm arena with the rear wall being projected at. This wall contained both the target, a square of 42 cm x 70 cm and the background, spanning over the whole wall. For both colours a contrast ratio between the background and the target of 0.4 and 0.6 was needed to evoke a significant response. Experiment II was conducted in a virtual reality (VR) set-up called SpiderVR. The untethered spider was put on a 2D treadmill that compensated for its movements. The surrounding VR provided a seamless 360° projection (vertical about 50°) with a spatial resolution of less than 0.2° per pixel and a temporal resolution of 60 fps. Both spatial and temporal resolution of the set-up are higher than the resolution of the spider eyes and thus individual pixels and frames are not perceived separately.
The background was projected all around the spider, and one target was shown. As in experiment I the spiders showed a significant preference only for targets darker than the background. However, a contrast ratio of 0.25 was enough to evoke this behaviour.

Results show that *Cupiennius salei* only approaches targets darker than the background. An intensity contrast between the background and the target of at least 0.25 is needed to evoke a solid response. Therefore it is suggested, that this behaviour rather relies on contrast than on colour vision.

Key words: colour vision, behavioural test, spectral sensitivity, virtual reality, *Cupiennius salei*, spider, vision

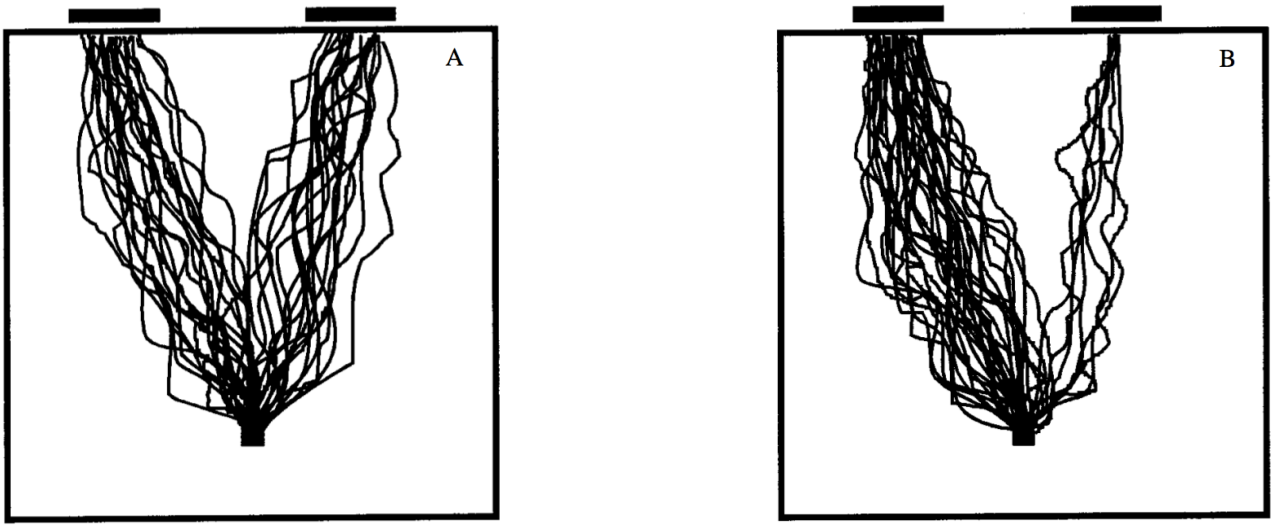
1 Introduction

1.1 Animals

The hunting spider *Cupiennius salei* has its origin in Central America and can be found in the neotropical rainforest of Mexico, Guatemala and Honduras at an altitude of 200 to 1250 m above sea level (Barth and Seyfarth, 1979; Barth, 2002). It belongs to the family of wandering spiders (Ctenidae). This spider does not use a web but is a nocturnal sit-and-wait hunter. The prey mainly consists of insects (Melchers, 1967). During the day it seeks shelter in different monocotyledons such as agaves and banana plants (Barth and Seyfarth, 1979). Male spiders reach a leg span of more than 10 cm and are in general smaller than females (Barth, 2002).
It was shown that airflow cues, simulating flying insects, are enough to trigger the spiders prey capture jump and neither vision nor vibrational cues are needed (Melchers, 1967; Klopsch et al., 2012, 2013). Though this spider strongly relies on mechanical cues, the visual system is highly developed too (Land and Barth, 1992). Recently, it could be shown that *Cupiennius salei* also jumps for prey only perceived by visual cues (Fenk et al., 2010; Schützinger, 2014).
There is a long tradition of using *Cupiennius salei* for physiological, behavioural and neurobiological questions (Barth, 2002; Uetz and Roberts, 2002). In recent years the visual system caught more and more attention (Fenk et al., 2010; Fenk and Schmid, 2010, 2011; Orlando and Schmid, 2011; Lindner, 2013; Zopf et al., 2013; Campione and Schmid, 2014; Schützinger, 2014).

1.2 Behaviour

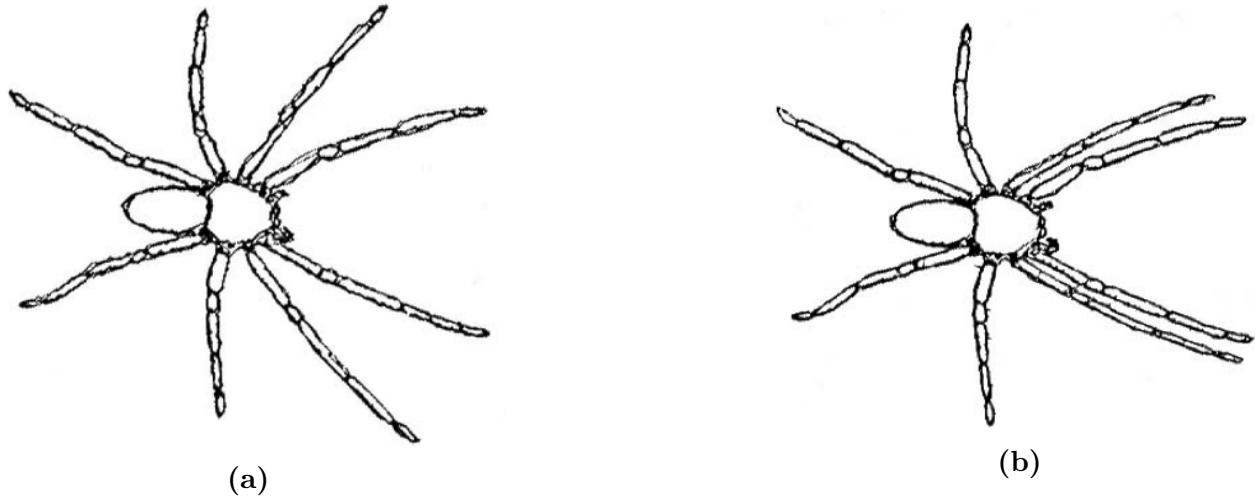
The male spiders are more active than the females. The sexually motivated active searching behaviour for females was suggested as a reason (Schmitt et al., 1990). During the day and most of the night they like to hide on plants, taking shelter at the base of big leaves, sitting with there prosoma orientated downwards. During this time, *C. salei* is only occasionally active and, if so, in the early morning hours. The main activity occurs after sunset (Barth and Seyfarth, 1979).
The distinct courtship behaviour of males, where they stop walking and start to move their opisthosoma dorsoventrally to elicit vibrations of the substrate, can be observed regularly. If a female perceives this signal it responds the same way but with lower frequencies (Rovner and Barth, 1981; Barth, 1997).



(a) **(b)**

Figure 1: Paths of spiders walking towards two targets (a) both targets are vertical squares (b) right target is tilted, showing a preference for non tilted targets (Schmid, 1998).

As *C. salei* spends most of its adult live on plants such as banana plants, it is essential for the spider to find those if it has fallen to the ground. From the ground, trees look like vertically orientated squares. Schmid (1998) showed that *C. salei* prefers such vertically orientated black bars on white background over tilted bars (figure 1). Lots of research was done on this target orientated walking behaviour making it to one of the major assays for behavioural research in *C. salei* (Neuhofer et al., 2009; Theil, 2010; Zopf et al., 2013).
Noticeable is the fright posture, where *C. salei* presses its body to the ground and aligns the two frontal pairs of legs in a certain way (figure 2b). This is often shown when the soil is suddenly vibrating distinctly or something moves in the visual field. In contrast, figure 2a shows the normal leg posture of a spider with the body lifted from the ground.



(a) **(b)**

Figure 2: Different leg postures of *C. salei* (a) showing the normal and (b) the fright posture (Kaps, 1998).

1.3 Eyes

C. salei has four pairs of eyes arranged in two curved rows. The rear row consists of the posterior median (PM) and posterior lateral (PL), and the frontal row of the anterior median (AM) and anterior lateral (AL) eyes (figure 3a). All eyes are circular shaped lens eyes and differ in size. The PM eyes are the largest, the AL eyes the smallest (Land and Barth, 1992). Compared to other terrestrial arthropods the eyes of *C. salei* are highly sensitive. Changes in the rhabdomal microvilli structure influence the sensitivity of the photoreceptor cells over the day resulting in a higher sensitivity during night than at daytime (Grusch et al., 1997). Due to morphological differences the AM eyes are also called principal eyes, the remaining three pairs are called secondary eyes. AM eyes do not have a tapetum and the structure of their photoreceptors are different (Land and Barth, 1992). The retina of the secondary eyes have inverse photoreceptor cells where the light first has to pass the cell body and then the rhabdomeres (Land and Barth, 1992). The retina of the secondary eyes is stationary, whereas the retina of the principal eyes can be moved by muscles. This movement leads to a displacement of the visual field of up to 15° (Land and Barth, 1992; Kaps and Schmid, 1996; Kaps, 1998; Barth, 2002). It was shown that small movements of the retina, so called microsaccades (amplitude of 2° - 4°), are responsible for preventing visual adaptation (Kaps and Schmid, 1996).

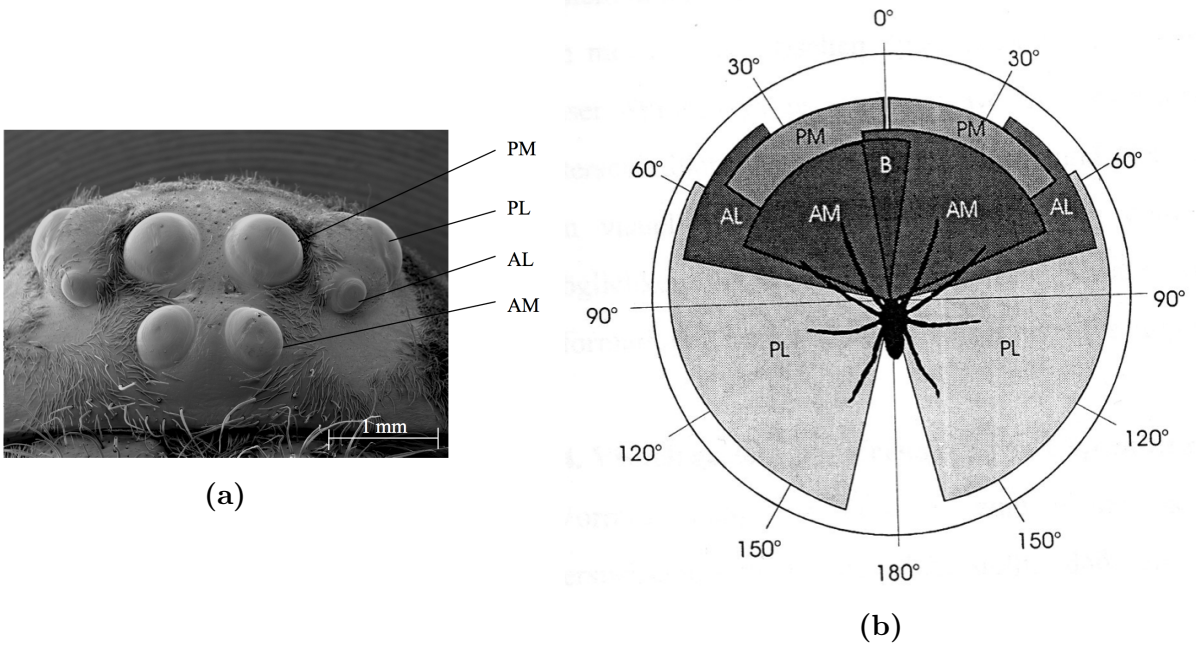


Figure 3: (a) Frontal SEM image of *C. salei* showing all eight eyes (Zopf, 2010); (b) horizontal field of view of an adult spider (Kaps, 1998); Eyes: posterior median (PM), posterior lateral (PL), anterior median (AM), anterior lateral (AL).

The receptors are distributed regularly over the AM retina, which results in a interreceptor angle of 2.9° (Land and Barth, 1992). The photoreceptors of the secondary eyes are arranged in distinct rows, and the spatial resolution differs in the horizontal (1° ; PM and PL eyes) and vertical (2-3° , PM and PL eyes) plane (Land and Barth, 1992; Fenk et al., 2010). The resolution of the AL eyes is 3.6° in the horizontal and 9.3° in the vertical plane (Land and Barth, 1992). From each eye an optic nerve projects to the first neuropile (Babu and Barth, 1984).

The PM and PL eyes cover almost the whole upper hemisphere and up to 40° below the horizon (Land and Barth, 1992). In the front the AM eyes show a narrow binocular region. Further, the forward orientated visual fields of the PM and AM eyes overlap almost completely suggesting different visual tasks (Land and Barth, 1992). All eight eyes provide the spider with an almost complete picture of its environment, except for a small gap in the rear where the abdomen would occlude the view anyway (Land and Barth, 1992)(figure 3b).
As suggested by these these differences in morphology, *C. salei* uses the different eye types for different aspects of visual processing. The secondary eyes are used for movement detection where principal eyes are not involved (Neuhofer et al., 2009). The AM eyes are used for target-discrimination, extracting features like orientation of a target, and the PM eyes are used for target-detection (Schmid, 1998). Specialisation of different eyes is not unique for *C. salei*, but was also shown for jumping spiders (Land, 1969; Duelli, 1978; Zurek et al., 2010; Menda et al., 2014).
For a long time it was believed that the visual system of spiders is not as important as their mechano-sensory system (Barth, 2002; Klopsch et al., 2012, 2013). Recent studies, however, show that *Cupiennius salei* reacts to visual cues alone too, underlining the importance of the visual system (Fenk et al., 2010; Lindner, 2013; Schützinger, 2014).

1.4 Brightness Discrimination

As a nocturnal hunter, *Cupiennius salei* has a very sensitive visual system. Electroretinograms suggest an absolute corneal luminance threshold of below 0.01 lx (Barth et al., 1993). Campione and Schmid (2014) showed that the dismantling of the rhabdomeric membrane during the day (Grusch et al., 1997) has an effect on the ability to discriminate contrasts. Spiders with night adapted eyes performed much better. A smaller Michelson-constrast (0.1 - 0.2) is sufficient to elicit a significant increase in AM eye muscle activity compared with day-adapted eyes (0.2 - 0.3). Eye muscle activity is used as a measure to decide whether the brightness steps are discriminated by the PM eyes.

1.5 Colour Vision in *Cupiennius salei*

The composition of light with different wavelengths is another feature of visual information. Even if two objects have the same brightness, they can be distinguished from one another if their reflected spectrum is different (Menzel, 1979). The concept of colour is composed of brightness, saturation and hue. Brightness is the achromatic aspect of colour and refers to the intensity and saturation describes the similarity of a colour to a neutral grey. Hue is the difference in colour, also referred by the names of the colours (e.g. red, green, blue, purple) and is a function of the spectral maxima of both, the signal and the observers sensor (Kelber et al., 2003). For discriminating wavelengths, so called "colour vision", two or more photoreceptor cells, acting as sensors, with different absorption maxima and overlapping spectral sensitivity have to be

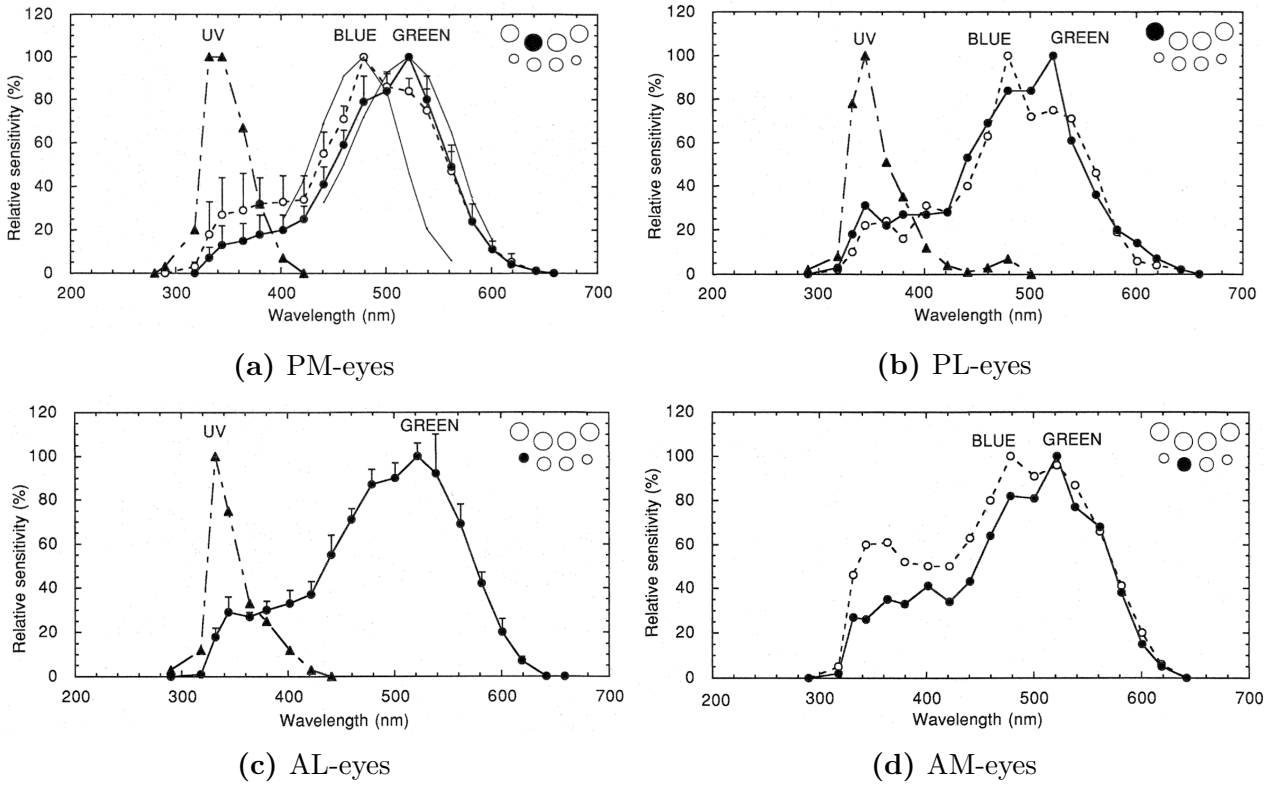
present. However, colour vision only is found in arthropods and vertebrates (Koyanagi et al., 2008). The photon sensitive structure in the cell is composed of an opsin protein and a chromophore (photopigment). Despite of the chromophore being the photosensitive structure, the opsin is responsible for the wavelength sensitivity. A photoreceptor cell can be seen as a photon counter, regardless of its spectral sensitivity, once a photon is absorbed, the response is the same. This means that a single type of photoreceptor cannot distinguish between wavelengths (Kelber et al., 2003).

The two modalities spatial and temporal resolution are linked inversely. The absolute sensitivity of the cells and the photons captured by the eye are the limiting factors. This leads to different approaches of adaptation (e.g. temporal or spatial pooling) to improve vision under dim conditions (Kelber et al., 2003). Pooling the colour information could be used to extend the spectral sensitivity and thereby detecting more photons without using colour as an additional quality (Warrant and Nilsson, 2006; Warrant and Dacke, 2011).

Koyanagi and colleagues (2008) suggest that the last common ancestor of living arthropods had at least three different opsins, two in the visible and one in the UV range. For *Cupiennius salei* three visual opsins were found for detecting light from UV up to longer wavelengths of the spectrum (Zopf et al., 2013). The spectral sensitivity of the eyes of *C. salei* has been investigated for a long time. Experiments concerning the global sensitivity of the eyes to monochromatic light were first conducted by Barth and colleagues (1993). By using electroretinograms a sensitivity from about 300 - 650 nm, with peaks at 520 - 540 nm and a shoulder in the UV range between 340 and 380 nm was shown. The UV peak was about 40% to 50% of the green peak. Conducting intracellular recordings of the photoreceptor cells revealed that *Cupiennius salei* has three different groups of receptor cells with their maximum in the UV (340 nm), blue (480 nm) and green (520 nm), respectively (Walla et al., 1996). Both the green and the blue sensitive receptor cell showed a second peak at the maximum of the UV receptor. In all but the AL eyes the majority of cells found were blue and green receptor cells of roughly equal proportions (figure 4). In the AL eyes the green sensitive cells dominated and no blue sensitive cell was found (figure 4c). UV cells were found only in the secondary eyes but not in the principal eyes (figure 4d). A reason for this, given by Walla and colleagues (1996), was that the moving retina of the AM eyes made it hard to record at all. This resulted in very few recorded cells and could imply that UV cells were overlooked. In the PM and PL eyes all three types of receptors were found (figure 4a and 4b). In general, the blue cells have a small shoulder at the peak of the green cells and the other way around, a reason suggested for this is electric coupling (Walla et al., 1996). Another reason could be the co-expression of several visual opsins in one cell which could be used to broaden the spectral sensitivity of the cell (Sakamoto et al., 1996; Kitamoto et al., 1998; Dalton et al., 2014; Isayama et al., 2014).
Zopf and colleagues (2013) demonstrated in a behavioural study that the spiders walked to a black stripe in a white arena illuminated by monochromatic light also when longer wavelengths

were used. This suggests that the sensitivity is slightly shifted to longer wavelengths compared to previous studies (Walla et al., 1996; Barth et al., 1993).

For the jumping spider *Hasarius adansoni* the presence of colour vision was shown in a behavioural experiment. The spiders were tested by a heat avoidance learning task in combination with coloured paper. It was shown that they can distinguish between blue, green, yellow and red (Nakamura and Yamashita, 2000).
Electroretinograms indicate that the nocturnal wandering spider *Leucorchestris arenicola* can not discriminate colour and only possesses one type of photoreceptor cells per eye (Nørgaard et al., 2008). Contrary to this finding up to four different photoreceptor cells were found in jumping spiders, ranging from UV (330 nm) up to deep red (700 nm) (Land, 1969; Yamashita and Tateda, 1976; Peaslee and Wilson, 1989). Two different cell types were found in wolf spider (Lycosids) eyes, although only in the principal eyes both receptor types (green and UV) were present, in the secondary eyes the UV receptor was missing (DeVoe, 1972).



(a) PM-eyes **(b)** PL-eyes

(c) AL-eyes **(d)** AM-eyes

Figure 4: Spectral sensitivity of single photoreceptor cells of the different eyes. (a) Posterior median eye: 1 UV cell (λ_{max} 340-380 nm) ,16 blue cells (λ_{max} 480 nm), 15 green cells (λ_{max} 520 nm). (b) Posterior lateral eye: 1 UV cell (λ_{max} 340 nm), 2 blue cells (λ_{max} 480 nm), 1 green cell (λ_{max}520 nm); (c) Anterior lateral eye: 1 UV cell (λ_{max} 335 nm), 5 green cells (λ_{max} 520 nm); (d) Anterior median eye: 2 blue cells (λ_{max} 480 nm), 2 green cells (λ_{max} 520 nm), (Walla et al., 1996).

It is suggested that the movement of the retina in the AM eyes is involved in the ability to detect stationary objects (Kaps and Schmid, 1996; Schmid, 1998). This was further investigated, showing that the activity of the eye muscles of the AM eyes increased if the secondary eyes perceived movement, but not when only the AM eyes see movement (Neuhofer et al., 2009).

This paradigm was used for the first approach of testing colour vision in *C. salei* by Orlando and Schmid (2011). Coloured strips were moved in front of several different grey backgrounds. The intensity of the background was changed from white to black in 28 steps. As a stripe moved in the visual field of the secondary eyes the activity of the AM muscles increased. The activity increase of the muscles dropped below significance for the blue, green and red stripe at a few similar grey steps. Therefore, the stripe was not discriminated from the background, and thus no chromatic mechanism is involved in motion vision.

1.6 Research Question

The question arose if a behavioural assay could be used for showing colour vision in *C. salei*. The presence of distinct types of photoreceptors with different spectral sensitivities suggests that chromatic cues may be processed by the spiders but is not enough on its own to prove it. Also it was shown that other nocturnal animals and also other spiders possess colour vision (Nakamura and Yamashita, 2000; Koyanagi et al., 2008; Warrant and Dacke, 2011). On the contrary, it was shown on tethered *Cupiennius salei* that the movement detecting system is colour blind (Orlando and Schmid, 2011). To prove that *Cupiennius salei* uses colour as an additional cue while navigating through the environment, colour vision needs to be tested in a behavioural context. This study investigates the ability of freely walking *C. salei* to discriminate a target from its background by colour, regardless of its intensity. The experiments thus aim at testing whether *C. salei* also uses colour as environmental information.

2 Materials and Methods

2.1 Animals

For the experiments adult male spiders of the species *Cupiennius salei* were used. They were bred and raised at the Department of Neurobiology (University of Vienna). The animals were kept separately in glass jars (r = 7.5 cm, h = 25 cm) with an average relative humidity of 71 % and an average temperature of 25° C. Feeding with *Calliphora sp.* took place once a week, water was available at all time. At least three days before testing an individual spider, it was set on a controlled 12-12 hour day-light cycle.
The reason for using only male spiders was that the draglines created by females contain a sex pheromone which would influence male spiders in subsequent experiments (Tichy et al., 2001).

2.2 Experiment I - Arena Colour Projection

2.2.1 Experimental Set-up

The arena is an adapted version of the arena (210 cm x 210 cm) described by Zopf and colleagues (2013) and the same target-size (42 cm width x 70 cm height) was used. Instead of placing a target made out of cardboard at the wall, we projected it with a consumer LCD-projector (Sony VPL-CX70, Sony, Konan Minato, Tokyo, Japan) to the rear wall as illustrated in figure 5a. The only light source was the projector. All walls and the floor of the arena were white. The projection consisted of a background and the target, both being projected to the rear wall of the arena (figure 5). A combination of a background with a target of the opposite colour is called a condition.

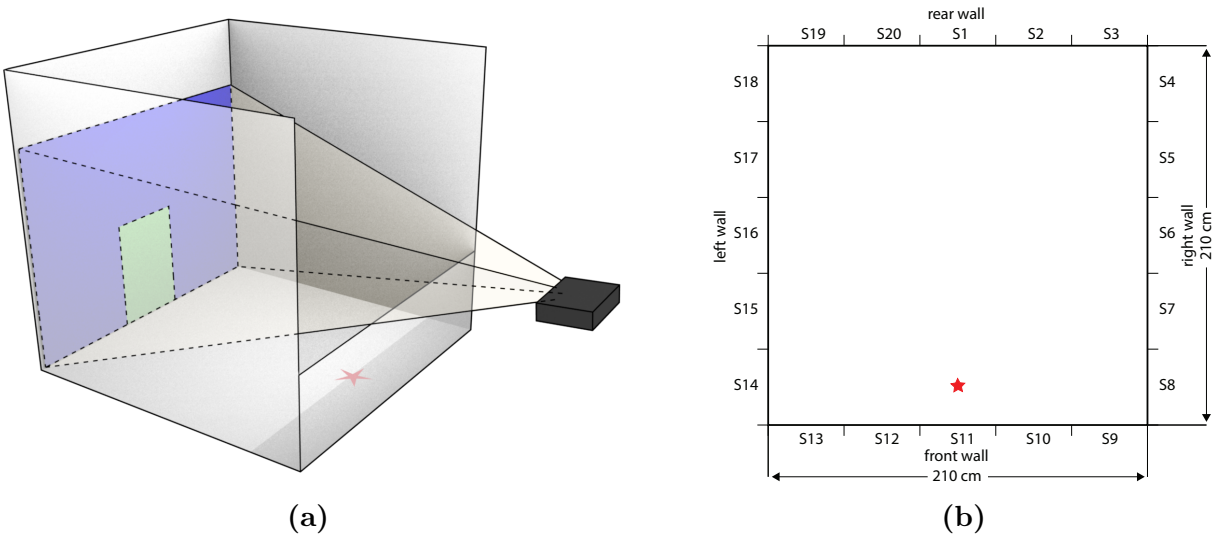


Figure 5: (a) Illustration of the arena, with condition k7 displayed, the target (green square of 42 cm x 70 cm) and the background were always projected on the rear wall. (b) top-view of the arena with the walls divided in sectors. The target was always displayed in sector S1, the spider was released in front of sector S11 facing in the direction of the rear wall. The asterisk indicates the starting-point for all spiders.

2.2.2 Experimental Protocol

The tests started approximately 2 hours after the onset of the night phase for the spiders, and took at maximum three hours. Each spider was tested two times at maximum. First, the spider was moved from the glass jar to a plastic cup. Then, the spider was placed on the starting-point in the arena (red star in figure 5a). As soon as it oriented itself towards the rear wall the plastic cup was lifted and therefore the spider was released. Afterwards, the spider was captured and stored in the plastic cup for the second run. The condition was not changed until all spiders completed the first run. Spiders were started in random order. This order was maintained for the second run. Spiders that were not moving in three attempts were excluded from all experiments and any data of their aborted runs was excluded.

2.2.3 Target

In sum 13 different conditions were tested. For generation of the projection Microsoft Powerpoint was used. The target was an upright rectangle of either blue or green colour on a background with the respective other colour. The projected rectangle had a width of 42 cm and height of 70 cm (the same size as in previous experiments; Schmid, 1997; Zopf et al., 2013). The intensity of the target was varied with R-G-B values (reaching from 0 to 255 for all three colours respectively) while leaving the background at full intensity. All 16 testing-conditions with the resulting intensities and contrast values are shown in table 1.

Table 1: Conditions used in the arena experiment. The intensity of the background stayed constant, the intensity of the target was changed. A combination of background and target is called condition.The intensity was weighted by the spectral sensitivity of the AM eyes of *Cupiennius salei* (ERG recordings; Walla et al. 1996) and the contrast was calculated using the Michelson-contrast (equation 1).

	intensity [photons/cm^2]			R - G - B		
target	background	target	contrast	condition	background	target
-	2.53×10^9	-	-	k0	0 - 0 - 252	0 - 0 - 252
blue	2.53×10^9	4.92×10^8	0.707°	k1	0 - 0 - 252	0 - 24 - 0
		6.18×10^8	0.681	k2	0 - 0 - 252	0 - 44 - 0
		9.93×10^8	0.603	k3	0 - 0 - 252	0 - 81 - 0
		2.36×10^9	0.404	k4	0 - 0 - 252	0 - 135 - 0
		3.90×10^9	0.202	k5	0 - 0 - 252	0 - 195 - 0
		3.91×10^9	0.198*	k6	0 - 0 - 252	0 - 252 - 0
green	4.15×10^9	6.65×10^8	0.713°	k7	0 - 252 - 0	0 - 0 - 0
		7.12×10^8	0.674	k8	0 - 252 - 0	0 - 0 - 24
		7.87×10^8	0.607	k9	0 - 252 - 0	0 - 0 - 44
		1.03×10^9	0.436	k10	0 - 252 - 0	0 - 0 - 81
		1.76×10^9	0.034*	k11	0 - 252 - 0	0 - 0 - 138

° max contrast *min contrast

2.2.4 Intensity Measurements

The intensity of the different conditions was measured with a calibrated spectral photometer (JAZ, Ocean Optics, Tokyo, Japan). It was measured as irradiance of a white diffuse cosine corrector by the projection. The sensor was placed 30 cm in front of the rear wall in a 45° angle to prevent shadow casting and direct irradiation. The spectral photometer was facing the position where the target would appear. The sensor was not moved during the whole series of measurements to get comparable readings. To measure the background, only the background was projected, to measure the target both were projected. Measurements where available as absolute irradiance (µWatt/cm^2/nm), converted to photons/cm^2/nm and then weighted by the spectral sensitivity of the AM eyes of *Cupiennius salei* (Walla et al., 1996). The intensity was calculated by summing up this arbitrary scaled spectrum from 300 nm to 700 nm. The contrast was defined as a modification of the Michelson-contrast by

$$\frac{I_{BG} - I_{TR}}{I_{BG} + I_{TR}} \tag{1}$$

with I_{BG} and I_{TR} being the intensity of the background and the target. The advantage of this modification is that if the target is brighter than the background, it is indicated by a negative contrast.

2.2.5 Data Recording

Each wall was divided in 5 segments, all of the same size. Lines were drawn to the corresponding edge of the opposite wall, dividing the floor in 25 tiles (figure 5b). The rear wall, which was projected on, contained the target in the central segment. The target was of exactly the same width as one segment. The path of the spiders was recorded by hand and as soon as the spider touched a wall the segment was noted and the run was considered to be finished. If the spider did not move or stopped moving it was captured after 5 minutes. If the spider was running it was not stopped. Between the runs the spiders remained in the plastic cups to avoid handling stress.

2.2.6 Control

As it was not possible to illuminate all walls of the arena equally, a control experiment (k0) was carried out to test for spatial preferences. The rear wall was illuminated only with green light (same as condition k1-k6, but without the target).

2.2.7 Data Analysis

Based on the results of the control experiment (k0), where the rear wall without a target was avoided by the spiders (figure 11a and table 3), it was decided, to bin all runs towards a wall. This led to four categories: left, rear (with the projection), right and front wall. The distribution of runs in the control experiment (k0) was taken as a reference, and compared

to all other conditions. To test if the distribution of runs under a condition was different to the control experiment, both distributions were compared with a Monte Carlo simulation of a Chi-square test for goodness-of-fit with 10^6 replicates (McDonald, 2014). To figure out which wall was preferred in a test condition, the distribution of runs to one particular vs. all other walls (eg. left wall vs. all other walls) in the test condition was compared to runs to the same wall vs. all other walls in the control experiment. This was done with a Monte Carlo simulation with 10^6 replicates of a Chi-square test for goodness-of-fit (McDonald, 2014).
A p-value of 0.05 was applied as significance level and corrected using the Bonferroni correction. As there were five statistical tests applied (one initial test and four post-hoc tests), a corrected significance level of p = 0.01 was calculated. Analysis and statistical testing was done with Python (Python 2.7, www.python.org) and R (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org)

2.3 SpiderVR - Tracking and Locomotion Compensation

SpiderVR was developed in parallel to the Arena experiments and was used for the experiment II. SpiderVR (figure 6) is a combination of a 2D treadmill and a virtual reality (VR) allowing

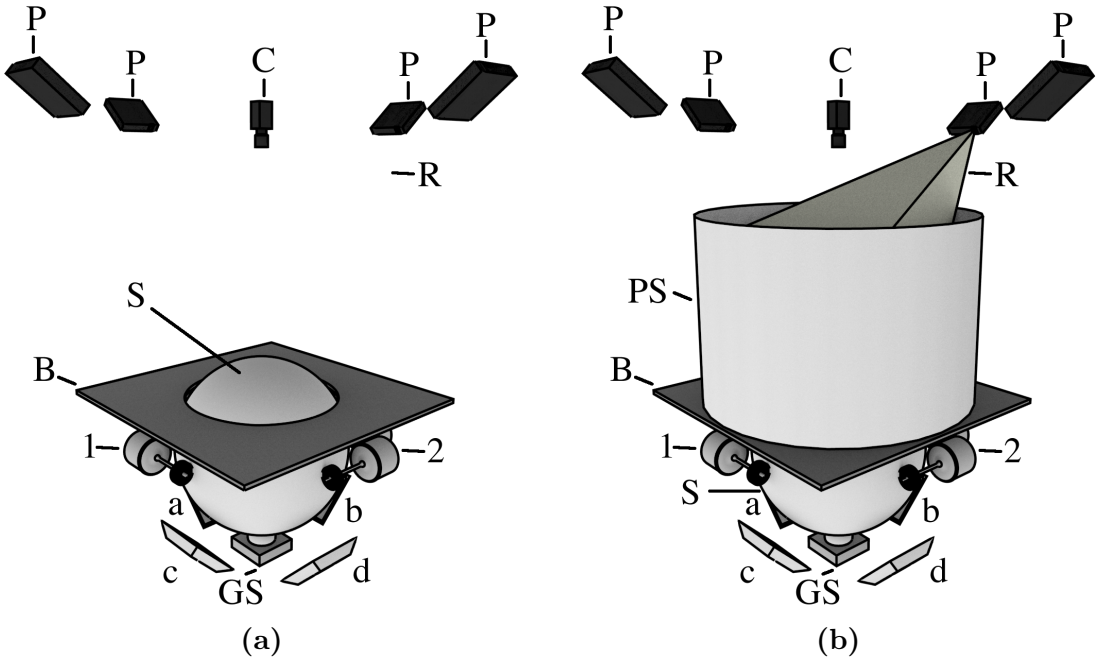


Figure 6: Schematic illustration of SpiderVR, a 2D treadmill with a surrounding virtual reality; parts from bottom up: four IR-LED panels (a,b,c,d), air supported glass sphere (GS), main sphere (S), four motors (two visible 1,2), board (B), lampshade as projection surface (PS, only in b), camera (C), four projectors (P, with projection light rays indicated only in b as R). The experimental animal was placed at the top of the sphere and was freely moving while a virtual reality was presented. When the spider was walking, the motors moved the sphere in the opposite direction, preventing it from displacement in any direction.

the study of visual guided behaviour in ground-dwelling animals like *Cupiennius salei*. In comparison to experiments where the animals have to be tethered, this experimental set-up does not require any manipulation of the used animal during the experiment. The VR can

simulate "real world" scenarios that can be manipulated in many ways. Brightness, contrast and colour can be altered, objects can be moved or resized and much more. This enables creating open-loop as well as closed-loop experiments.

2.3.1 Mechanical Components

The sphere is the centerpiece of the treadmill. Its basic mechanical design is a prototype of the Servo-Ball from Phenosys GmbH (Phenosys GmbH, Berlin, Germany) and was adapted for the use with spiders. It is made from an opaque synthetic material coated with small particles to provide good friction. It has a diameter of 60 cm and is hollow. It is resting on a smaller, air supported glass sphere to reduce friction while rotating. Four 120 Watt DC motors, two for each axis, are used to rotate the sphere in its frame. The rotation of the sphere is controlled by custom made software spiderTrackingVR v1.1 (Max Hofbauer 2014). On each axis a rotary encoder (A2-CW3C, Yumo, Yueqing City, China) is mounted to measure the exact distance the sphere had moved. The signal is processed by an Arduino UNO (www.arduino.cc) connected to a computer.
The **IR-light sources** (LED emitter with spectral peaks at 850 nm and 920 nm), with an overall power consumption of approximately 100 Watt, are placed below the sphere (figure 6). The light is scattered through the sphere's rough surface, which results in a homogeneous diffuse illumination of the sphere. A camera (ace602f-2, Basler, Ahrensburg, Gemany) was mounted on an overhead traverse and equipped with an IR-transmission filter (transmission starts at 730 nm). The spectrum of the LED's was outside the spectral sensitivity of *C. salei* (Schmid, 1997; Walla et al., 1996; Zopf et al., 2013) in order to not interfere with behaviour.

2.3.2 Software

The control software "spiderTrackingVR v1.1" was developed for this set-up by Max Hofbauer. The major aim was to control the sphere in such a way that the spider does not escape, even through at jumps or fast runs, but also not to disturb the spider with fast movements when it moves slowly. When the spider is moving slowly, abrupt acceleration could easily lead to fright-posture of the animal (figure 2b).

The camera used provides 8-bit greyscale images with 100 fps (frames per second) and a resolution of 600 x 480 pixel (figure 7a). These images are used to determine the position of the spider on the sphere. For image processing, routines of the open source Intel OpenCV library are used as described by Bradski and Kaehler (2008) and Yilmaz and colleagues (2006). First, a simple blob detection is applied, consisting of special image filtering procedures (gaussian blur, dilation, erosion, threshold), followed by structural analysis (contour detection, moments calculation). The largest contour, by means of area (up to a certain threshold), is assumed to be the spider and therefore chosen for further processing. The center of this contour is calculated

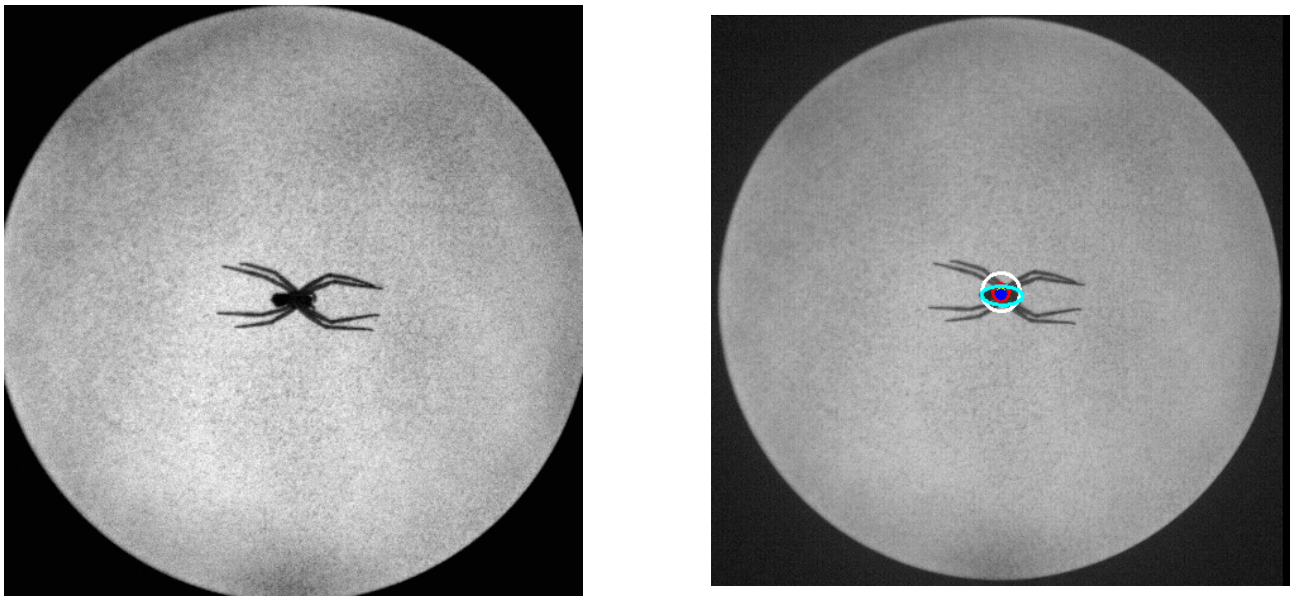
by

$$\bar{x} = \frac{M_{10}}{M_{00}} \tag{2}$$

and

$$\bar{y} = \frac{M_{01}}{M_{00}} \tag{3}$$

where M_{pq} is the moment of order (p + q) and $\bar{x}$ and $\bar{y}$ is the center of mass in a 2D coordinate space. The center of mass is the assumed position of the spider. Furthermore, an ellipse is fitted to this contour to estimate the orientation of the body axis of the spider. Due to the limited processing power of the PC that had to be used for computation, all functions were optimized to achieve a stable operating frame rate of 100 fps. This was also the reason not to include more power consuming image processing operations like camshift, cascade classifiers or even saving the video while tracking.



(a) **(b)**

Figure 7: (a) IR greyscale image of a spider on the sphere (600px x 480px, 8-bit) (b) Tracked spider at target position: the blue dot in the middle states the position of the spider, it was moved into the inner circle (target position). The outer circle defines the threshold at which the compensation starts to move the dot (spider) back to the target position. An ellipse was fitted to estimate the orientation of the spider.

To translate the position of the spider on the sphere to an adequate response of the motors a proportional-integral-derivative (PID) controller was integrated for each axis separately (Wescott, 2000). The resulting signal is sent to an analogue-digital converter connected to an amplifier. There the signal was translated to voltage values between -10 and +10 V controlling both the speed and direction of the motors. The initial tuning values were derived by the Ziegler-Nichols method for tuning PID controllers (Ziegler and Nichols, 1942). The advantage of this implementation is, that if the spider walks slowly, the sphere is only accelerating slowly and thus causes only minor vibratory disturbance. Whereas if the spider runs or jumps it im-

mediately compensates with its full power. Overshooting of the compensation in the opposite direction was also minimized by this procedure.

2.3.3 Virtual Reality - FlyVR

To generate the virtual reality (VR), four standard LED DLP-projectors (Odys Picto mini DLP-Projektor, AXDIA, Willich, Germany) with a resolution of 854 x 480 pixel were used. DLP-projectors use an array of micro mirrors instead of a LCD panel to produce a pixel and have a LED as light source. This has some advantages over common LED-projectors: the light is not polarized, the contrast is higher and the the size of the projector is smaller. A disadvantage is the colour-wheel "rainbow-effect". When fast moving objects are presented the edges split in red, green and blue shadows. Since the temporal resolution of the spiders eyes is lower than the response time of the projector this aspect should not be problematic (Fenk and Schmid, 2011).
As projection surface a white cylinder (d 90 cm, h 60 cm), a common lampshade, was placed around the sphere. Its lower edge is approximately 10 cm below the top of the sphere. The projectors are arranged in such a way that they projected crosswise to the opposite side of the cylinder, resulting in a theoretical pixel size of about 1 mm. The height of the projection on the lampshade was about 45 cm. Altogether this led to a seamless projection of 360° vertically and 50° horizontally with a resolution of less than 0.2°.

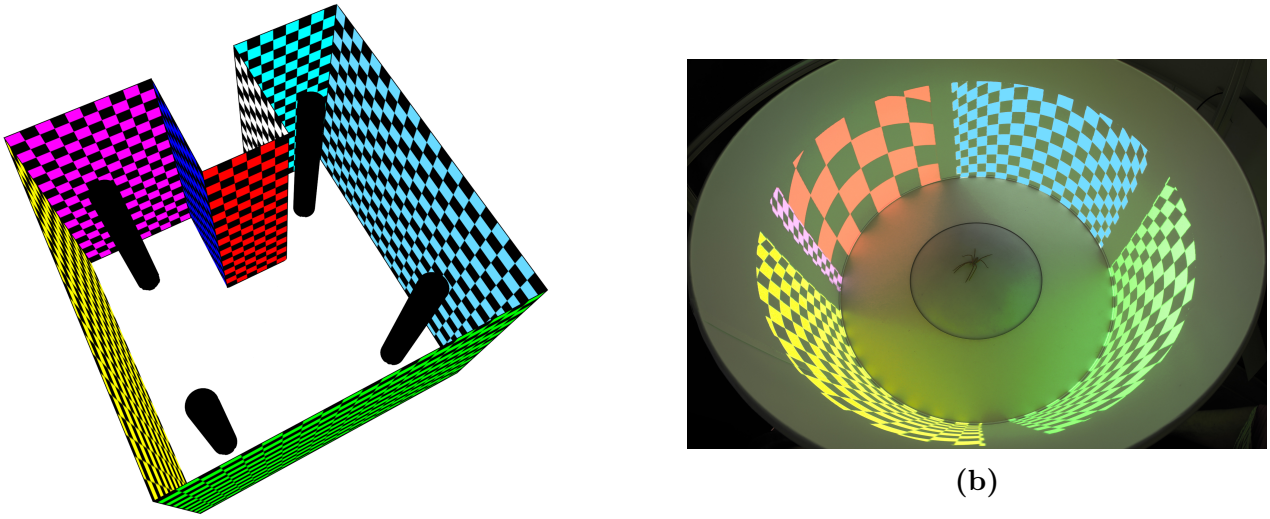
To generate an accurate visual impression, the open source package FlyVR (Stowers et al., 2014) available at www.flyvr.org was used. This software loads a given 3D environment and renders it for a viewpoint and a given projection geometry. In case of SpiderVR this is a cylinder. Both the position of the spider on the sphere, as well as the position of the sphere in the virtual environment are taken into account for the projection. If a spider walks in one direction in a virtual environment, and thereby the sphere is rotating in the opposite direction, the resulting position of the spider in the virtual environment is the same as in real world. The projection is also corrected for the current viewpoint of the spider.

2.3.4 Creating an Experiment

The process of creating a new experiment consists of the following steps: 3D modelling of the environment; programming experimental- and physical-constraints (eg.: the experiment is completed when a certain point is space is reached; is a wall solid or not) and logging.

3D Modelling

To generate an experiment the 3D model (figure 8a) of the virtual environment was generated using the open source 3D-modelling software Blender (Blender 2.67, Stichting Blender Foundation, Amsterdam, Netherlands, http://www.blender.org). The model is scaled in real world coordinates and the level z = 0 is assumed as ground level. As FlyVR uses OpenSceneGraph (www.openscenegraph.org) the 3D model needs to be converted to .osgt format.



(a) **(b)**

Figure 8: From the generated 3D model (a) to the projection (b). (b) is a photograph of the used set-up with the VR presenting the 3D model from (a) to a spider sitting on the sphere.

Experimental and Physical Logic

A python script (Python 2.7, www.python.org) was developed to work as a controller between the location data of the spider, FlyVR and the experimental protocol. The script loads the condition according to the experimental protocol to the VR and aligns the tracking position. Thereby certain constraints, such as the solidity of walls, position of objects (e.g. the targets in experiment II), position triggered events (e.g. loading next condition if the spider reached a target) or even the colour of objects could be altered.

First, experimental conditions were set, describing parameters like the starting position of the spider, maximum duration of trial and what happens if the spider reaches a certain position. These parameters were evaluated during the experiment and updated with the same rate as the tracking was running (100 Hz).

Logging

One of the most important parts of such a system is to save all the important data and associate it with the correct time-stamp. For this reason we decided to log all data, the tracking data (e.g. x,y position of the sphere in the 3D world, x,y of the spider on the sphere,) and the experimental data to one time-series. Instant plots of the spiders path were saved to control for errors in tracking or compensation.

2.4 Experiment II - VR Colour Projection

2.4.1 Experimental Set-up

For this experiment SpiderVR, as described in 2.3, was used to display different combinations of target and background with various contrasts and colours. The target was a vertically orientated rectangle either of the colour blue or green, while the background had the opposite colour. The test-conditions consisted of different contrast ratios of target and background.

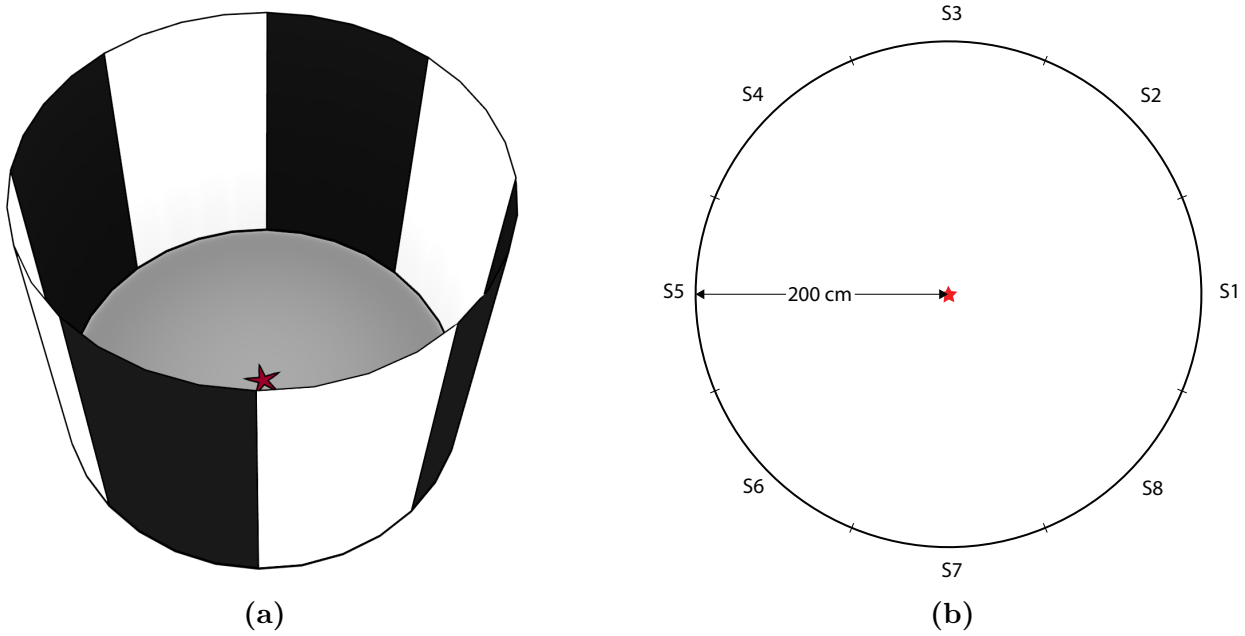
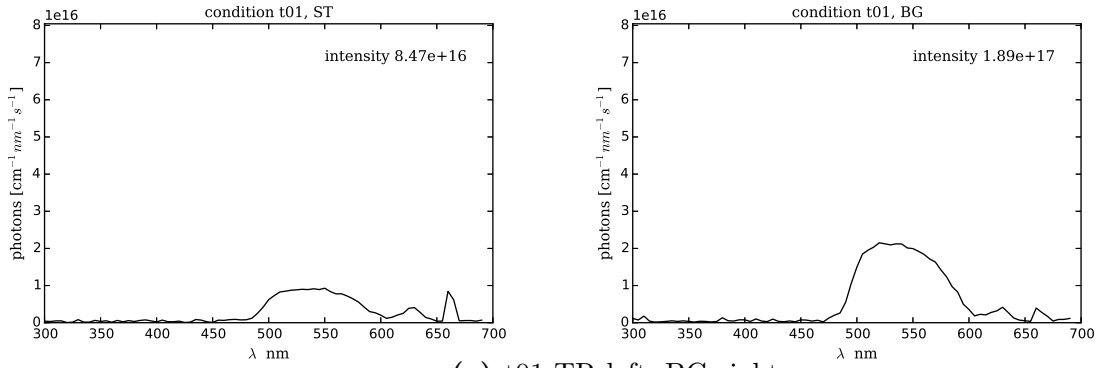


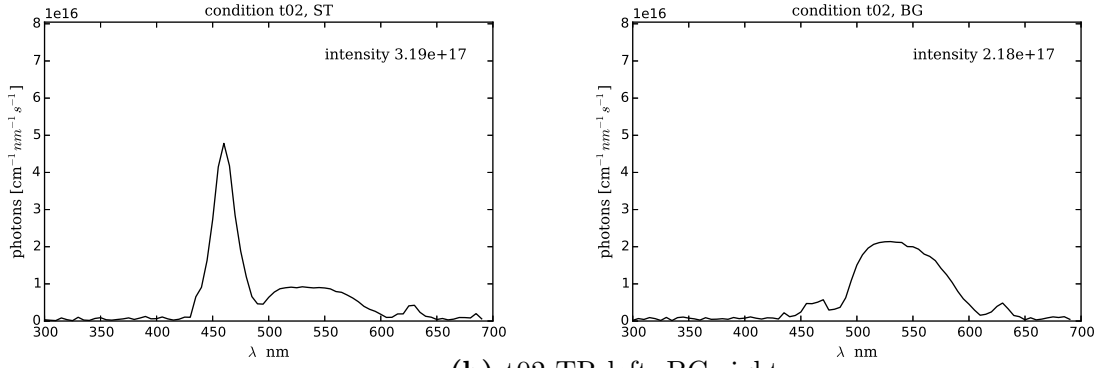
Figure 9: (a) Illustration of the virtual arena, displaying the starting condition (table 5, t001) with targets in all four display-sectors (s1, s3, s5, s7). While testing, the target is shown only in one of these four sectors. (b) Top-view of the arena with the walls divided in sectors. The target was randomly displayed in one of the display-sectors. The red star indicates the starting position of the spider.

2.4.2 Intensity Measurements

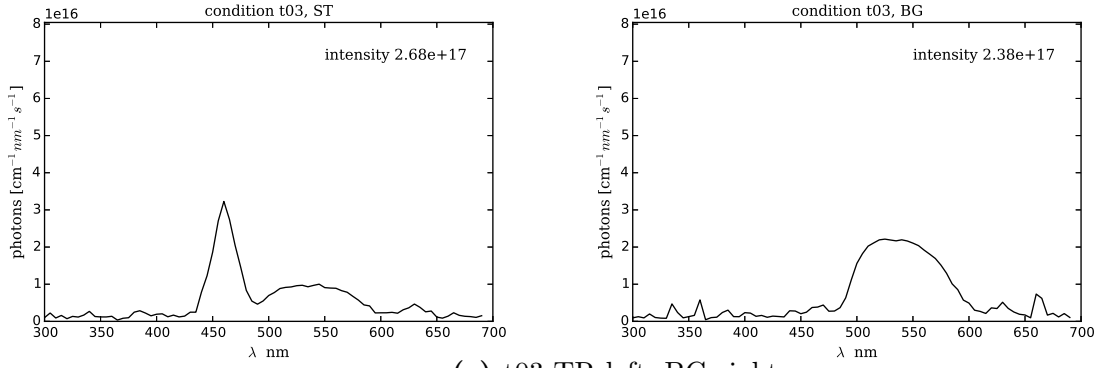
The intensity of the projection was measured with a photo spectrometer (ILT 900-R, International Light, Polytec, Waldbronn, Germany). To achieve a reliable measurement the sensor was placed 10 cm in front of the projection surface in a 45° vertical angle. This prevented shadow casting and direct irradiation. The sensor was moved between measurements. In order to ensure comparable measurements, the target was displayed in the segment in front of the sensor for measuring the target intensity and projected to the sector of the opposite side (180°) for measuring the background intensity. The resulting spectra (figure 10) were then weighted by the spectral sensitivity of the AM eyes of *Cupiennius salei* (Walla et al., 1996). The intensity was calculated by summing up this arbitrary scaled spectrum from 415 nm to 650 nm. The narrower range, compared with experiment I was chosen to reduce the influence of noise, which was higher with the used spectrometer in experiment II. Since the projector does not emit light in the UV-range, omitting this range from the calculation does not alter the results. The contrast was calculated with a modified Michelson-contrast (equation 1).



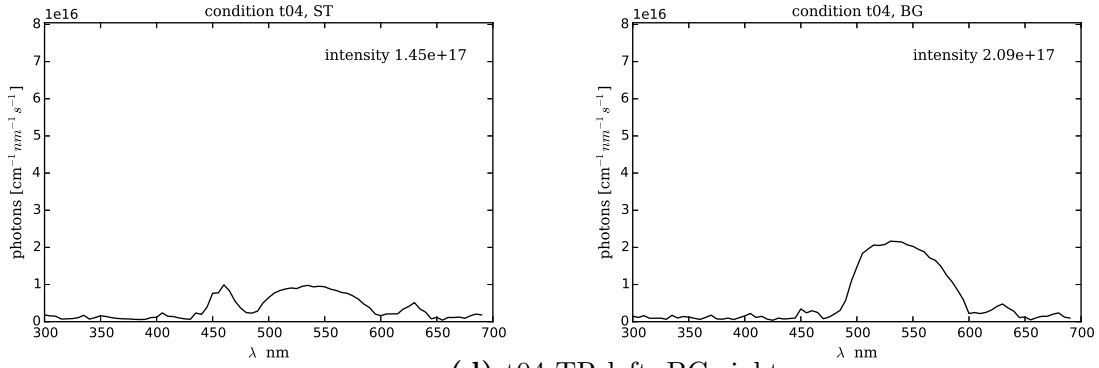
(a) t01 TR left, BG right



(b) t02 TR left, BG right



(c) t03 TR left, BG right



(d) t04 TR left, BG right

Figure 10: Spectra of conditions t0 - t04 (a - d, target blue, background green) as described in table 2. Measurements of the target are shown on the left (TR) and measurements of the background on the right side (BG). Spectra of all remaining conditions can be found in the appendix (figure 13).

Due to reflections, the background influences the spectrum of the target and vice versa. This is shown in figure 10 and 13. The bump between 450 nm - 480 nm in figure 10b-right indicates

the presence of the target. The corresponding spectrum of the target is shown in the plot on the left side. In comparison, this bump is missing in figure 10a-right. This is because under this condition the target is black, and therefore no blue colour is projected. The same principle applies for the measurements of the target.

2.4.3 Experimental Protocol

Experiments took place during the day. In a testing session the spider was placed on the sphere of SpiderVR with a plastic cup covering it. As soon as the cup was lifted, the tracking and compensation started. The experimental control program was started as soon as possible. When the spider did not start walking or did not pass the start-condition (t001) the spider was exchanged. If a spider did not wove for several trials it was removed from the pool of spiders. Each test session consisted of the start-condition and 16 test-conditions separated by the break-condition. The period from the start of one condition till the start of the next condition is called run.

2.4.4 Conditions

As soon as the experiment started a virtual cylindrical arena with a diameter of 400 cm was presented around the spider (figure 9). The wall of the arena was split every 45° leading to eight segments of the same size. Stimuli were only displayed in every second segment resulting in four display-segments (s1, s3, s5 and s7). The spider was placed in the center of the arena at the start of each condition (red star in figure 9). The resulting distance to the wall of the arena was 200 cm.
A session started with the start-condition. This was followed by 16 test-conditions in random order. Between these conditions the arena was dark for 30 s to calm down the spider (break-condition).

Start-condition
The start-condition had to be passed successfully to proceed to testing. It was used as control experiment to test if the spider responded in the anticipated way. All four display-segments were black, the others were white. This resulted in the maximum contrast between target and background. If the spider walked into any segment, and therefore had to walk at least 2 m the start-condition was successfully passed, otherwise the spider was replaced.

Test-conditions
Under the test condition, seven of the eight segments had the colour of the background and could not be distinguished from each other. The remaining segment had the opposite colour and thereby formed the target. The colours green and blue were used to generate either the background or the target. If the target was green, the background was blue. The spiders were tested in two modes, a bright one, where the target was brighter than the background (bright target), and a dark one, where the target was darker (dark target). For both modes

four modifications were generated with a constant background intensity and a changing target intensity, chosen from the range of intensities between isoluminant with the background and black (dark condition) or full intensity (bright condition). This was carried out for both colours likewise and resulted in a total of 16 test-conditions (table 2). The intensity of the target was chosen in such a way that both the maximum contrast under the bright-condition and the maximum contrast under the dark-condition were similar and in addition the contrasts were similar for both colours. Because blue was the limiting factor by means of intensity (figure 13e-right in the appendix and table 2 condition t09), the maximum contrast was set by projecting a blue background and a black target. The resulting contrast was 0.303 and is shown in table 2. If a spider did not reach the wall within 300 s, a timeout was triggered. This lead to a break-condition, followed by the next test-condition.

Break-condition

A break was presented between each of the other conditions. During this condition the whole arena was dark and no visual stimulation took place. The duration was 30 s regardless of the behaviour of the spider.

Table 2: Conditions used in the VR set-up and their intensities and contrasts

traget		intensity [photons/cm^2/s]				R - G - B	
colour	mode	background	target	contrast	condition	background	target
black	dark	1.14×10^{18}	2.56×10^{15}	0.98°	t001	1 - 1 - 1	0 - 0 - 0
blue	dark	3.27×10^{17}	1.54×10^{17}	0.358°	t01	0 - 0.57 - 0	0 - 0 - 0
		3.51×10^{17}	3.32×10^{17}	0.029*	t02	0 - 0.57 - 0	0 - 0 - 1
		3.66×10^{17}	2.95×10^{17}	0.106	t03	0 - 0.57 - 0	0 - 0 - 0.60
		3.44×10^{17}	2.01×10^{17}	0.263	t04	0 - 0.57 - 0	0 - 0 - 0.35
	bright	9.88×10^{16}	2.26×10^{17}	-0.391°	t05	0 - 0.3 - 0	0 - 0 - 1
		8.43×10^{16}	8.79×10^{16}	-0.021*	t06	0 - 0.3 - 0	0 - 0 - 0.4
		1.56×10^{17}	2.03×10^{17}	-0.130	t07	0 - 0.3 - 0	0 - 0 - 0.55
		1.13×10^{17}	2.08×10^{17}	-0.295	t08	0 - 0.3 - 0	0 - 0 - 0.70
green	dark	3.53×10^{17}	1.89×10^{17}	0.303°	t09	0 - 0 - 1	0 - 0 - 0
		3.88×10^{17}	3.83×10^{17}	0.006*	t10	0 - 0 - 1	0 - 0.57 - 0
		3.87×10^{17}	3.15×10^{17}	0.102	t11	0 - 0 - 1	0 - 0.43 - 0
		3.31×10^{17}	2.18×10^{17}	0.207	t12	0 - 0 - 1	0 - 0.35 - 0
	bright	1.08×10^{17}	2.59×10^{17}	-0.409°	t13	0 - 0 - 0.4	0 - 0.57 - 0
		1.09×10^{17}	1.21×10^{17}	-0.053*	t14	0 - 0 - 0.4	0 - 0.35 - 0
		1.39×10^{17}	1.80×10^{17}	-0.130	t15	0 - 0 - 0.4	0 - 0.42 - 0
		1.47×10^{17}	2.44×10^{17}	-0.249	t16	0 - 0 - 0.4	0 - 0.51 - 0

°max contrast *min contrast

2.4.5 Data Analysis

Data analysis was carried out on the log files of SpiderVR. As the target was shown in sector 1, 3, 5 or 7 by chance, the trajectories of the runs under the test-conditions were rotated in

such a way, as if the target would have been displayed in sector S1. Afterwards, all runs to S1 were runs to the target. For statistical analysis a Pearson's Chi-square test for goodness-of-fit with a Monte Carlo simulation with 10^6 replicates was used (McDonald, 2014). To test if the distribution of runs in a test-condition differed from a uniform distribution, a Monte Carlo simulation of a Chi-square test for goodness-of-fit with 10^6 replicates was applied (McDonald, 2014). To figure out if a segment had more counts than expected by chance, the count of runs to and not to a segment were compared the expected values. This was done with a Monte Carlo simulation with 10^6 replicates of a Chi-square test for goodness-of-fit (McDonald, 2014). A p-value of 0.01 was determined as significance level. As there were nine statistical tests applied on each condition a corrected significance level of $p = 0.0011$ was calculated following the Bonferroni correction.

3 Results

3.1 Experiment I

In total 750 runs were started of which 533 could be used for analysis. The remaining 217 runs had to be aborted (spider did not walk or stopped walking) and were therefore not used for analysis. After releasing a spider in the arena it typically needed a few seconds to start walking. The first part of the run was often characterized by turning on, or around the starting point. If approaching the target, most spiders aimed for the edges of the target and not the center.

As shown under the control condition k0 where no target was presented and the rear wall was illuminated by green light of full intensity (figure 11a), the runs were not equally distributed over all four walls ($p \leq 0.001^*$ table 3). The rear wall was avoided and especially sector s7 and s8 seemed to be preferred. This is reflected in all test conditions as well. In general, the spiders showed the typical zigzag mode, where they turn their body axis left and right while approaching their target. This was also observed under conditions where the overall response was not significant (k4, k5, k6, k10 ,k11). Although the geometry of the trajectory was only recorded by hand, runs to the target under these conditions appeared orientated and over all very similar to runs under conditions with significant results..

The relative distribution of runs over all sectors is shown in figure 11 as histograms. The target was always displayed in sector S1. Most positive runs were recorded for condition k10 and k11, 55 respectively. The lowest number of positive runs was recorded under condition k2. For both target colours the spiders showed a significantly different distribution of reached sectors than under the control condition (k0) up to a similar contrast of 0.6 and 0.4 (table 3). Testing which wall was preferred revealed that under all significant conditions the rear wall was preferred (table 4). In addition, under condition k1 the right wall showed significant less runs than expected (expected 15.5 runs). Condition k4, even though being not significantly different from the control-condition, shows a significantly elevated count at the rear wall (table 4).

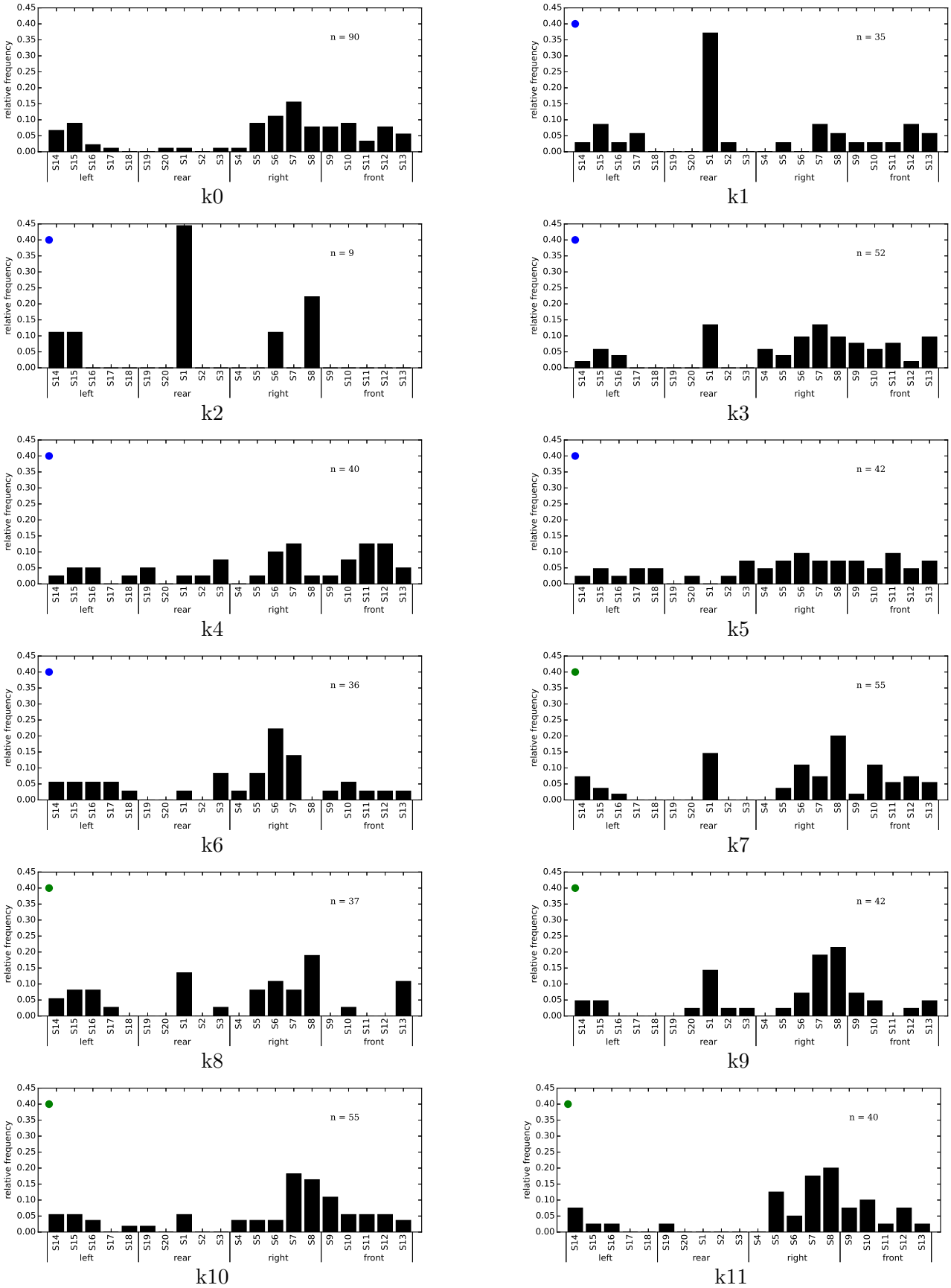


Figure 11: Relative distribution of positive runs to the according sector; the target was presented in S1. (k0) control experiment with no target (target was identical with the background), the whole rear wall was green, (k1) - (k6) target blue, background green; (k7) - (k11) target green, background blue; the contrast between the target and background decreased from k1 - k6 and from k7 to k11.

Table 3: The distribution of runs to the walls under the test conditions (k1 - k11) were compared to the distribution expected by the control experiment (k0). The distribution of k0 was tested for equality. A Monte Carlo simulation with 10^6 replicates of a Pearson's chi-squared test was applied. Runs in test condition k1 - k3 and k7 - k9 were distributed significantly different in comparison to the control experiment (k0).

		wall [nr. of runs]					
target colour	condition	left	rear	right	front	contrast	p
-	k0	17	3	40	30		$p \leq 0.001^*$
blue	k1	7	14	6	8	0.707	$p \leq 0.001^*$
	k2	2	4	3	0	0.681	$p \leq 0.001^*$
	k3	6	7	22	17	0.603	$p \leq 0.001^*$
	k4	6	7	11	16	0.404	0.006
	k5	8	5	15	14	0.202	0.547
	k6	9	4	17	6	0.198	0.099
green	k7	7	8	23	17	0.713	$p \leq 0.001^*$
	k8	9	6	17	5	0.674	$p \leq 0.001^*$
	k9	4	9	21	8	0.607	$p \leq 0.001^*$
	k10	9	4	25	17	0.436	0.036
	k11	5	1	22	12	0.034	0.495

$^*p \leq 0.01$ significance level after Bonferroni correction

Table 4: To test which wall was preferred, a post-hoc test for difference in runs to the walls was conducted. Runs to a single wall vs. all remaining walls of a test condition were compared with the distribution in the control experiment. For statistical testing a Monte Carlo simulation of a Pearson's chi-squared test with 10^6 replicates was applied.

		wall [p value]			
target colour	condition	left	rear	right	front
blue	k1	1.000	$p \leq 0.001^*$	$p \leq 0.001^*$	0.213
	k2	1.000	$p \leq 0.001^*$	0.740	0.069
	k3	0.215	0.002^*	0.783	1.000
	k4	0.559	$p \leq 0.001^*$	0.037	0.402
	k5	1.000	0.013	0.280	1.000
	k6	0.392	0.032	0.868	0.050
green	k7	0.302	$p \leq 0.001^*$	0.787	0.776
	k8	0.401	0.001^*	0.870	0.013
	k9	0.166	$p \leq 0.001^*$	0.535	0.070
	k10	0.732	0.110	0.893	0.776
	k11	0.326	1.000	0.204	0.739

$^*p \leq 0.01$ significance level after Bonferroni correction

3.2 Experiment II

In total 84 sessions, each consisting of 17 conditions, were started. Due to spiders not moving and therefore not passing the start-condition, or technical issues, 28 sessions had to be excluded partially (technical issues that affected only one test-run) or entirely. In total 863 test-runs, spread over all test-conditions were recorded. In 46% the spider did not reach the wall before the timeout occurred. In general the running behaviour looked similar to that of experiment I and also the zigzag mode could be observed. Figure 12 shows the trajectory of all runs recorded under the start-condition (t001). Under this condition the background was white and four equal stimuli were presented in black. The negligence of sector s7 in comparison to the three remaining stimuli arose from the way the spiders were placed on the sphere (see also figure 9). Due to physical limitations, the spiders were manoeuvred on the sphere coming from sector s7 leading to an initial orientation towards sector s3. Overall a strong behavioural response to the stimuli is shown under this condition, also the average distance of 3.83 ± 2.36 m (mean ± s.e.m) is shorter compared to all other conditions (5.11 ± 3.76 m).
When the target was darker than the background the spiders showed a good response, independent of the background colour. The response stopped for both colours between a contrast ratio of about 0.2 and 0.1 (table 5). No significant response could be found for contrast ratios below that. This also applies for all conditions in which the target was brighter than the background. The proportion of timeouts was similar over all conditions and ranged from 31% under condition t05 to 66% in t14.

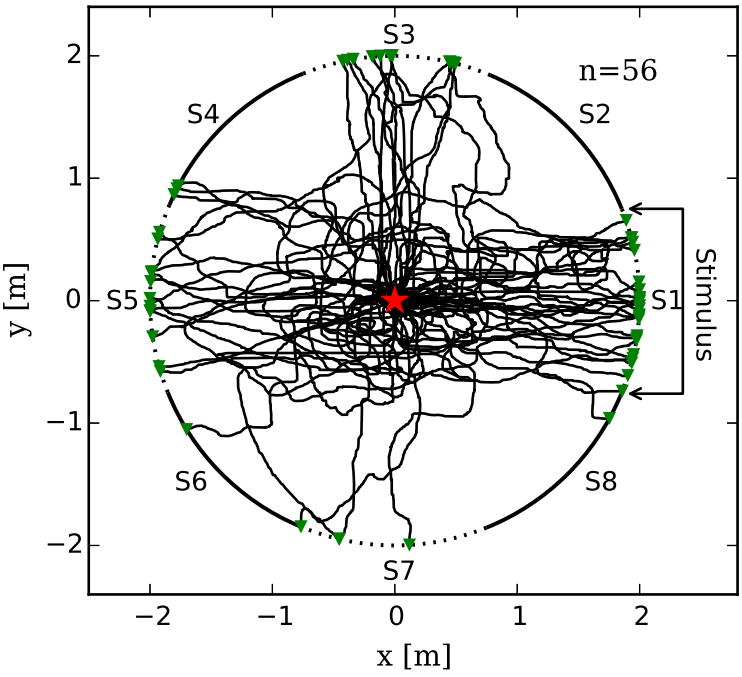


Figure 12: Condition t001. Dotted lines of the circle indicate where the black targets were presented, solid lines indicate where the white background was presented. To pass the test condition, a spider had to reach the wall (dotted or solid line). The red star marks the starting point for all runs (coordinates: 0 m , 0 m) and the green triangles mark the endpoints of each run.

Table 5: Statistical test for uniform distribution of runs to the segments, showing that the runs where not distributed evenly if the target was darker than the background (t01, t04, t09, t12). Tested with a Monte Carlo simulation of a Chi-square test for goodness-of-fit with 10^6 replicates. n is the sum of runs where the spiders reached a sector. °Sector number after rotation.

target			Sector°[nr]									timeout			
colour	mode	condition	1	2	3	4	5	6	7	8	n	total	did not move	mean distance [m]	p
black	dark	t001	23	0	11	3	13	2	3	1	56	-	-	3.83	$p \leq 0.001^*$
blue	dark	t01	31	2	2	1	22	0	3	0	92	31	21	5.16	$p \leq 0.001^*$
		t02	6	1	7	1	3	0	5	4	47	20	4	6.55	0.061
		t03	2	1	5	1	8	3	5	1	46	20	5	6.74	0.052
		t04	15	2	2	2	8	3	1	1	54	20	6	6.66	$p \leq 0.001^*$
	bright	t05	3	2	5	6	7	4	5	3	51	16	3	5.84	0.751
		t06	2	3	4	3	2	2	4	5	54	29	7	6.32	0.934
		t07	5	1	6	5	2	2	4	1	49	23	4	6.41	0.318
		t08	2	7	8	2	0	3	4	3	55	26	8	6.42	0.057
green	dark	t09	11	2	0	0	12	1	1	1	48	20	7	4.66	$p \leq 0.001^*$
		t10	1	2	4	2	2	6	2	3	52	30	10	7.72	0.543
		t11	5	2	1	0	7	4	1	4	53	29	9	8.19	0.068
		t12	14	0	6	1	5	0	0	2	55	27	7	6.11	$p \leq 0.001^*$
	bright	t13	0	6	6	2	9	3	4	3	53	20	6	5.44	0.067
		t14	6	2	0	4	1	0	1	3	51	34	11	6.18	0.046
		t15	2	3	7	5	2	1	7	0	51	24	7	6.31	0.040
		t16	2	2	5	7	1	1	7	7	52	20	5	5.71	0.061

$^*p \leq 0.001$ significance level after Bonferroni correction

Table 6: Post-hoc test for testing if the number of runs to one sector was different than expected by chance. It is shown that for condition t01 and t12 both, sector 1 and 5 are preferred and for condition t04 and t12 sector 1 is preferred over all other sectors. A Monte Carlo simulation with 10^6 replicates of a Pearson's chi-squared test for goodness-of-fit was used.

target			Sector							
colour	mode	condition	1	2	3	4	5	6	7	8
blue	dark	t01	$p \leq 0.001^*$	0.030	0.030	0.010	$p \leq 0.001^*$	0.003	0.081	0.003
		t02	0.140	0.245	0.044	0.245	1.000	0.071	0.376	0.768
		t03	0.572	0.244	0.366	0.244	0.011	1.000	0.365	0.244
		t04	$p \leq 0.001^*$	0.309	0.309	0.309	0.065	0.617	0.117	0.117
	bright	t05	0.615	0.308	0.796	0.438	0.196	1.000	0.797	0.616
		t06	0.575	1.000	0.762	1.000	0.575	0.574	0.762	0.358
		t07	0.365	0.243	0.128	0.364	0.572	0.571	0.765	0.244
		t08	0.425	0.083	0.023	0.426	0.043	0.794	1.000	0.794
green	dark	t09	$p \leq 0.001^*$	0.570	0.076	0.076	$p \leq 0.001^*$	0.248	0.248	0.248
		t10	0.351	0.763	0.513	0.762	0.763	0.048	0.762	1.000
		t11	0.352	0.760	0.353	0.111	0.024	0.761	0.352	0.761
		t12	$p \leq 0.001^*$	0.076	0.248	0.247	0.570	0.076	0.077	0.570
	bright	t13	0.029	0.427	0.426	0.311	0.017	0.620	1.000	0.620
		t14	0.014	1.000	0.155	0.258	0.509	0.155	0.510	0.714
		t15	0.569	1.000	0.044	0.374	0.569	0.244	0.044	0.071
		t16	0.423	0.423	0.792	0.174	0.174	0.174	0.174	0.174

$^*p \leq 0.001$ significance niveau afters Bonferroni correction

4 Discussion

The visual capabilities of spiders have been in the focus of research for quite some time. The aim of the present thesis however was, to investigate colour vision in *Cupiennius salei* in a behavioural task. On several occasions it has been shown that this species shows a distinct attraction for squared shaped vertical targets when released in an illuminated arena (Schmid, 1997; Neuhofer et al., 2009; Zopf et al., 2013). This behaviour was used in the presented work to test for colour vision in a behavioural context. The target was modified in such a way, that the spiders would have to use chromatic information to discriminate it from the background. The results indicate that intensity contrast is more important then colour, due to the fact that spiders only run for targets darker than the background. This suggests that *Cupiennius*, although equipped with the morphological structures necessary for colour vision, does not use colour as cue for finding squared shaped vertical targets.

The reasons for choosing this particular behaviour as basis for the experiments were deliberate. As these spiders eat only once a week, it is hard to conduct experiments with food rewards. Also, a heat avoidance paradigm, similar to that used to show colour vision on jumping spiders, did not show any conclusive results in *Cupiennius* (Nakamura and Yamashita, 2000; personal communication Axel Schmid). It is suggested that *Cupiennius salei* might not memorize and learn at all and therefore learning experiments, as conducted in several other species, were no option (Frisch, 1914; Kelber, 2010). The only well established behaviour was the running behaviour for targets (Schmid, 1998; Neuhofer et al., 2009; Zopf et al., 2013). It was therefore chosen for the present experiments. Previously, this behaviour was also used to investigate the spectral sensitivity (Zopf et al., 2013). These experiments show that the behavioural response to the target can be used to assess whether the object can be detected by the spider.
All prior experiments used dark targets on bright background, mimicking trees as shelter. However it would also be reasonable that bright targets of a similar shape can indicate the presence of a shelter and spiders benefit from detecting a bright vertical structure in an environment that misses any other structure. Therefore, both brighter and darker objects are used in the experiments.

The natural habitat of *C. salei* is the neotropical rainforest. Sensitivity for more than one wavelength could help to distinguish objects or structures not only by contrast of intensity, but also by contrast of colour. *C. salei* shows a spectral sensitivity in the region 300-650 nm (Barth et al., 1993). Furthermore, it has been shown that three different photoreceptors with spectral sensitivity maxima in the UV (340 nm), blue (480 nm), and green (520 nm) are present in the retinae of *C. salei* eyes (Walla et al., 1996). In the current experiments, video projected images are used. The projectors use the three (human-) colors red, green and blue. Since the spectral sensitivity of *C. salei* does not reach into the (human-)red region, only the green and blue video signal could be used. UV-projection was not possible with the current set-up. Furthermore, it is suggested that UV cells are only located in distinct areas of the retina and

were not found in the AM eyes, which excludes UV form object-detection (Walla et al., 1996). Also, *C. salei* performed worse when UV light was used in previous experiments testing for absolute intensity (Zopf et al., 2013).

Cupiennius salei is attracted by a black cardboard target presented on a wall that is illuminated by a global light source or a projector (Schmid, 1998; Zopf, 2010). The first question was if *C. salei* would respond to projected targets. To test this, condition k1 of the first experiment was introduced. Under this condition the background was projected in green at full intensity and the target was projected black. The experiment revealed that the spiders are attracted by the projected target and therefore confirmed the applicability of a projected background-target combination for testing colour contrast.

A problem that arose with the use of a projection to a single wall was reflection. Light dispersion and reflection of the rear wall caused an intensity gradient in the arena of the first experiment. The rear wall was the brightest (100%). The right and left wall showed a gradient of brightness, having the highest intensity in the corner with the rear wall (44%) and the lowest before the corner with the front wall (21%). The front wall showed a homogeneous brightness of about 24%. Also the target was effected by reflections. The black target under condition k1 was the dimmest with an intensity of 27% of the background, meaning that it was brighter than the left and right wall at the corner with the front wall. Nevertheless the spiders showed a strong attraction for the target under this condition. In contrast, the spiders where repelled by the rear wall if only the background was presented without a target (k0). With increasing brightness of the target, spiders showed an increasing interest in the region of the lowest intensity. This suggests that *Cupiennius salei* is not only attracted by the darkest area of the arena by means of absolute intensity, but also by the area of the highest contrast. Although using a very similar lighting situation as in previous experiments, these experiments did not show similar attraction to the corners, nor the repulsion of the rear wall if no target was presented (Zopf, 2010). An explanation could be that the intensity used was lower or higher, or that the projector did also project to the left and right walls. However, it was shown that the contrast needed to elicit a behavioural response in this experiment is similar to prior electro-physiological studies investigating the minimal contrast between two green areas by measuring the eye muscle activity of the AM eyes (Campione and Schmid, 2014).

It was not possible to solve the problem of uneven lighting arising from a projection in this arena. Two of the four walls were not movable and the geometry of the arena - a square - always causes problems with uneven brightness in the corners. To avoid this problem a circular projection surface was used for the second experiment. SpiderVR has no corners but uses a circular lampshade as projection surface. Also the way the projectors are aligned produces an almost even distribution of light around the spider. Compared to the arena of the first experiment the maximum contrast was much lower in SpiderVR. This arose also from the reflections,

however they were reflected equally over the whole area, not producing any regions with high intensity differences.

In the first experiment, only targets darker than the background were presented, while in the second experiment, an additional mode with brighter targets was introduced (table 2). It is shown that the threshold for an attractive target in the second experiment lies between a contrast ratio of 0.1 and 0.2, and thereby is lower than in the first experiment (contrast ratio between 0.4 and 0.6; see table 4 and table 6). On the contrary, if the target was much brighter than the background, the spiders seemed to be repelled (see figure 14 t13 in the appendix). Conditions with higher contrast ratios (t1 and t9) in the second experiment not only show significantly more runs to the sector where the target was located, but also to the sector exactly opposite to the target (see table 6 and figure 14 t1 and t9 in the appendix). Although the set-up was meant to prohibit light reflection, this sector could have been darker than the immediate neighbours, and therefore attractive. Comparing intensity measurements of the background of similar conditions with these conditions show that there is no difference in the background brightness when the target changes intensity (see table 2 and figure 10 t1 - t4 and figure 13 t9 - t12 in the appendix).

Both experiments show that the attractiveness of the target drops at a certain contrast when the target gets brighter but still is darker than the background. In both experiments this happened for either colours at roughly the same contrast ratio (table 3, table 5). This could be linked to previous data, where similar global sensitivity to monochromatic green and blue light was shown (Barth et al., 1993). It could also mean that the postulated green and blue receptor are not different, in fact, their spectral sensitivity is similar (Walla et al., 1996). As proposed, they could be electrically coupled, or both receptors could co-express both opsins. Such a linkage was shown for several species and leads to improved sensitivity under dim conditions (Sakamoto et al., 1996; Kitamoto et al., 1998; Dalton et al., 2014; Isayama et al., 2014). It was also shown that the motion detection system of *Cupiennius salei* confuses moving stripes of the colour green and blue with grey backgrounds of roughly the same relative reflectance (59% blue, 42% green, Orlando and Schmid, 2011). This evidence rises the question whether or not *Cupiennius salei* possesses a blue and a separated green photosensitive cell, or if there is only one photosensitive cell for this part of the spectrum. Also it could be that they use either the blue or the green channel, or pool the information from both cells in this task.

As *C. salei* is a nocturnal hunter, colour vision could have vanished in favour of other modalities of vision. This spider has a relatively low temporal resolution, but high spatial resolution especially in comparison to other nocturnal spiders (Fenk and Schmid, 2010, 2011). The first increases, the second reduces the ability to see under dim conditions. An additional strategy to increase the overall brightness sensitivity, essential under dim conditions, would be to combine the signal from different receptors to give an achromatic signal (Kelber et al., 2003; Warrant

and Dacke, 2011). Such strategies depend on the environmental and behavioural context. In bees and goldfish it has been shown that colour vision can be limited to certain behaviours (Menzel and Backhaus, 1989; Neumeyer, 1991). The suggestion that *Cupiennius salei* has photoreceptor cells sensitive for different wavelengths could be considered as an indication for the ability of colour discrimination.

Recently, it was shown that *C. salei* uses vision as a second modality for attack behaviour (Fenk et al., 2010; Lindner, 2013; Schützinger, 2014). Spiders jumped towards a vertically moving black dot on a green computer screen. Although it is suggested that the motion detection system of *C. salei* is colour blind, this attack behaviour could be used to verify this hypothesis by moving coloured dots on different backgrounds similar to the experiments by Orlando and Schmid (2011). The ecological relevance to capture prey regardless of its contrast to the background is given, supporting that colour vision might be relevant for this particular behaviour.

In summary it could be shown that contrast and intensity influence the attractiveness of a target. But nevertheless colour vision could not be excluded. The typical zig-zag mode of walking of *C. salei* observed in prior experiments was also observed in both experiments of this study (Schmid, 1997; Zopf et al., 2013). In this behaviour spiders sway their body axis to the left and right while approaching a target to induce motion parallax. This could be a mechanism to facilitate object detection (Schmid, 1998). Data suggests that *C. salei* uses this modality for distance discrimination, to detect which target is closest and run for it (Lehnert, 2011). Furthermore this behaviour was observed in all conditions of this study. It could mean that the spiders always perceived the target, even when it had the same intensity as the background or even was brighter. To test if colour vision plays a roll in this behaviour the set-up of the second experiment could be adapted to provide motion parallax cues based on colour contrasts. If *C. salei* perceives the object it could be expected that the orientation of the zig-zag mode would be directed to the object more often than to other parts of the arena. As this behaviour is mediated by the AM eyes it would be an additional paradigm for testing colour vision on those eyes.

Several challenges hampered the smooth implementation of the VR experiment, necessitating lots of testing and development. The main problem was that the purchased tracking for the locomotion compensator was not working as expected. The Servo-Ball (Phenosys GmbH, Berlin, Germany) was initially developed for rodents and was assumed to work on spiders that have roughly the same size. But compared to rodents, spiders reflect a lot of IR-light, causing the tracking to fail because of a lack of contrast. This issue was solved by adding more IR-light and placing the lamps beneath the sphere. With this set-up the sphere was bright and the spiders contrasted better. As a result an exact silhouette of the animal was obtained. Unfortunately the long legs and comparatively small body (opisthosoma and prosoma) of the spider negatively influenced the stability of the tracking causing the sphere to exhibit jerky

movements. The underlying reason was the way the image processing was implemented. The silhouette of a rodent does not change much when it moves and therefore the estimated center of the contour is stable. The change of the position of the spiders legs while walking has a dramatic influence on the contour picked up by the image processing algorithm. This results in a noisy estimation of the real center of the spider. To solve this problem a number of special image filtering procedures (gaussian blur, dilation, erosion, threshold) were implemented in a custom made program to remove the spiders legs from the contour. This allows to estimate the position of the spider much better.
Other problems arose concerning the compensation. The compensation-speed of the sphere was a linear function of how far away the spider was from the center. As a result, when adding speed the compensation was pulsatile, which frightened the spider. Reducing the speed led to low gain of compensation which made it easy for the spider escape from the sphere. All these challenges made it necessary to develop a custom tracking and compensation software.

Another problem encountered on the way to a working VR was that the DLP-projectors had a build-in autocorrection function that changed the input image to maximize contrast automatically. To project a dark blue square on a dark green background the projector maximized the contrast by increasing the intensity of both. This issue was fixed by dying the clipped, unused parts of the projection in bright red. This should, to the best knowledge of the author, have only little or no influence on the spider as the spectral maximum of the red provided by this projectors is at 634 nm. *C. salei* lacks photoreceptor cells sensitive for this wavelength, and behavioural responses could not be excited with monochromatic light above 599 nm (Walla et al., 1996; Zopf et al., 2013).
Another technical issue was that the sensors of the purchased set-up that measure the rotation of the sphere were reporting wrong values (e.g. movement, where no movement occurred). This was solved successfully by replacing the IR-mouse sensor by mechanical rotation encoders, directly attached to the driving shaft of one motor for each axis. The new encoders also had problems. Although the sphere was not moving, one encoder measured that it was moving at full speed for a short period of time (100 ms). Presumably this occurred when the encoder remained in a position between two ticks (these encoders work on the base of a light barrier and a wheel with holes), and therefore the signal is fluctuating. As a consequence some runs had to be excluded from the results.

SpiderVR provides the angle of the main body axis based on an ellipse fitting. Unfortunately, this data turned out to be not very reliable due to heavy noise. Since the shape of the detected blob (see section 2.3.2) was highly influenced by the shape and posture of the spider, the measured angle of the body axis was effected similarly. As mentioned before, the reason why this simple method was used is the limitation of the hardware that had to be used. To improve body axis measurement, a second image analysis pathway could be used, that operates on a lower frame rate and priority than the main pathway. Also adding more sophisticated filtering,

such as Kalman filtering could improve the signal. Orientation data of high accuracy could reveal if spiders that were originally repelled by a bright target gazed longer at the contrast edges than they would have done by chance. This could serve as a measure for whether the spiders could detect the object/edge independent of its attractivity.

A behaviour that could be interesting for future experiments was observed while designing the experimental set-up for the second experiment. While developing the described colour vision paradigm several tests were conducted with different virtual environments. In experiments with virtual walls that the spider could walk through (e.g. figure 8a) it was often observed that the spider stopped immediately after walking through the wall, turned around and walked again through the wall. The next response was similar to the walking behaviour in complete darkness. Under such circumstances the spider walks on six of its eight legs, using the first pairs as guide-sticks (Schmid, 1997). In the case of a virtual wall, it walked parallel to the wall, changing sides several times and waving its frontal legs at the supposed wall. This conflict situation, where the visual system suggests something different than the tactile system is very interesting and could be used to investigate the interplay between the sensory modalities. Therefore is could be tested which system has priority for the resulting behaviour.

To summarize, it could be shown that a working VR system (3D modelling, rendering, projection) for terrestrial arthropods can be readily built from consumer parts and open source software. Some problems during the implementation occurred, but they could be solved successfully. The conducted experiments (experiments 2) further show that the Spider VR can be used for behavioural experiments with large hunting spiders., and opens the field for a large variety of experiments.

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6 Appendix

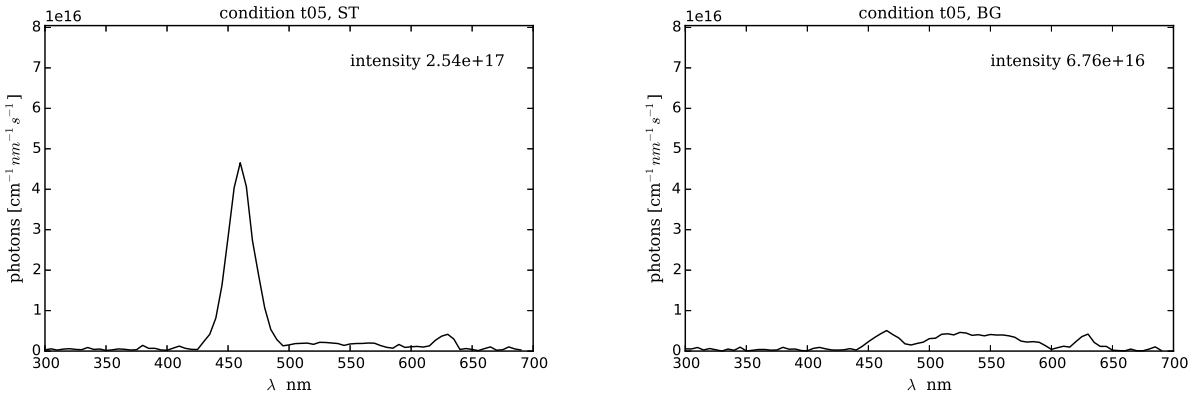
6.1 Deutsche Zusammenfassung

Das visuelle System der nachtaktiven Jagdspinne *Cupiennius salei* Keys, ist einer der am besten untersuchten Sinne bei Spinnen. Diese Spinne hat acht Augen, die sich in zwei Reihen angeordnet, in der frontalen Region des Prosomas befinden. Elektroretinogramme und intrazelluläre Ableitung zeigen eine breite spektrale Sensitivität die von etwa 300 nm bis 650 nm reicht. Diese Bandbreite wurde auch in Verhaltensexperimenten bestätigt. Die vorliegende Studie beschäftigt sich mit der Frage ob *C. salei* Targets (Rechtecke) nur durch Verwendung der Farbinformation von einem einfarbigen Hintergrund unterscheiden kann. Die Spinnen zeigen ein ausgeprägtes Interesse an vertikalen Strukturen und laufen auf diese zu. Es wurden sowohl blaue Targets auf grünem Hintergrund, als auch grüne Targets auf blauem Hintergrund in verschiedenen Intensitäten präsentiert. Die Verhaltensantwort auf diese beiden Konditionen wurde in zwei verschiedenen experimentellen Aufbauten getestet. In beiden Fällen wurde sowohl das Target und der Hintergrund mittels Projektoren projiziert und deren Kontrastverhältnis verändert.

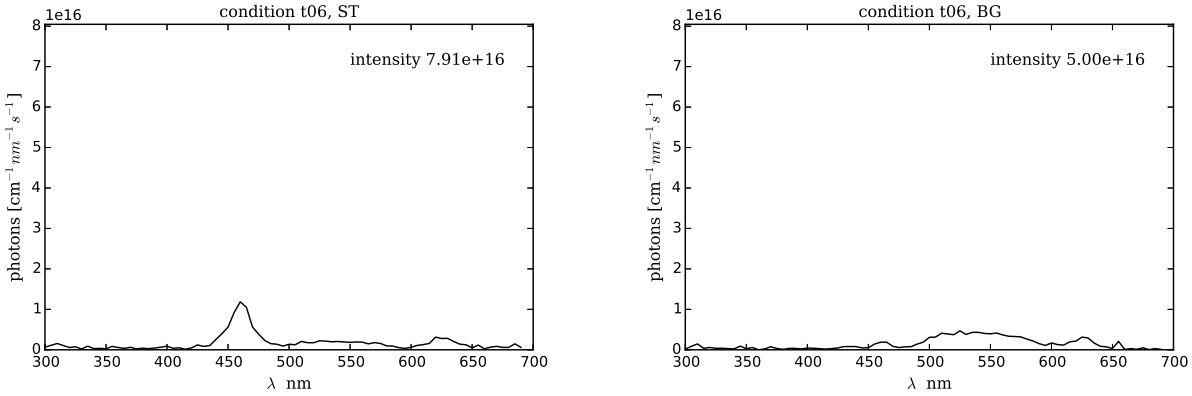
Experiment I wurde mit frei laufenden Spinnen in einer 210 cm x 210 cm großen Arena durchgeführt. In dieser Arena wurde der Hintergrund und das Target (Breite 42 cm, Höhe 70 cm) an die Hinterwand projiziert. *Cupiennius salei* brauchte einen Kontrast von 0.4 (Target blau) bzw. 0.6 (Target grün) um das Target signifikant öfter als die anderen Bereichen der Arena anzulaufen.

Im Rahmen von Experiment II wurde eine Virtuelle Realität (VR) mit einem Laufkompensator entwickelt (SpiderVR). SpiderVR ermöglicht es einer frei laufenden Spinne virtuelle Stimuli zu präsentieren, wobei die Spinne auf einer Kugel platziert wird und durch eine Kompensationsbewegung immer an der gleichen Stelle gehalten wird. Die die Spinne umgebende Projektion ist homogen und umfasst 360° horizontal und 50° vertikal. Die Größe eines einzelnen Pixels im visuellen Feld der Spinne beträgt weniger als 0.2° und ist somit unterhalb der Auflösungsgrenze ihrer Augen. In Experiment II wurde um die Spinne herum ein virtueller Zylinder mit einem Durchmesser von 400 cm simuliert, der in acht gleich große vertikale Segmente geteilt wurde. Jeweils sieben dieser Segment wurden in der Farbe des Hintergrunds gefärbt und eines in der des Targets. Wie in Experiment I waren Targets nur dann attraktiv wenn sie dunkler waren als der Hintergrund. Jedoch war schon ein Kontrast von 0.25 ausreichend um dieses Verhalten auszulösen.
Wenn Farbsehen in diesem Verhalten eine Rolle spielt, müsste *Cupiennius salei* das Target in allen Konditionen sehen, und, wenn sonst keine Struktur gegeben ist, auf dieses zulaufen. Dies konnte in den hier durchgeführten Versuchen nicht bestätigt werden, somit wird angenommen, dass in diesem Verhaltenskontext Farbwahrnehmung keine Rolle spielt. Des weiteren wird angenommen, dass dieses Verhalten durch Kontrastwahrnehmung zustande kommt, und helle Targets kein attraktives Ziel darstellen.

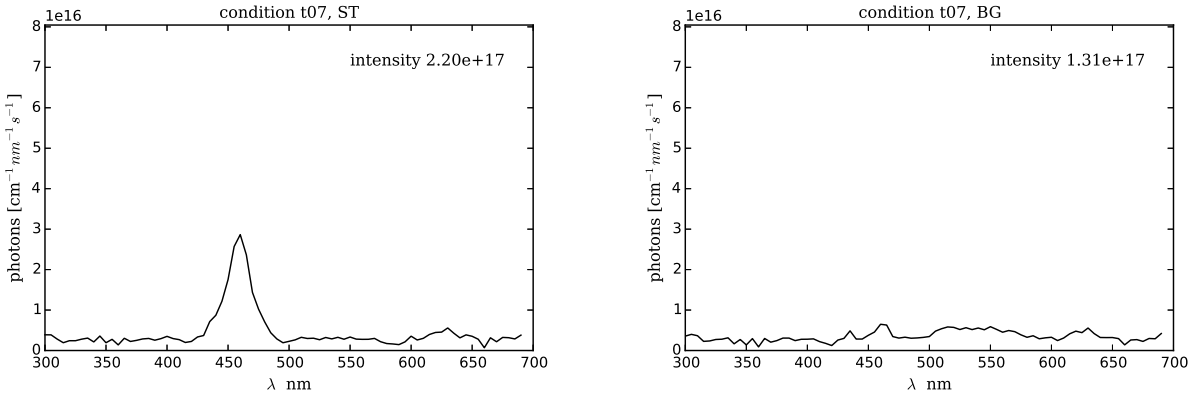
6.2 Additional Plots



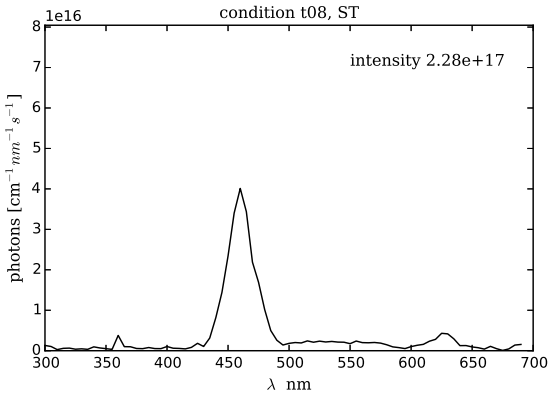
(a) t05 TR left, BG right

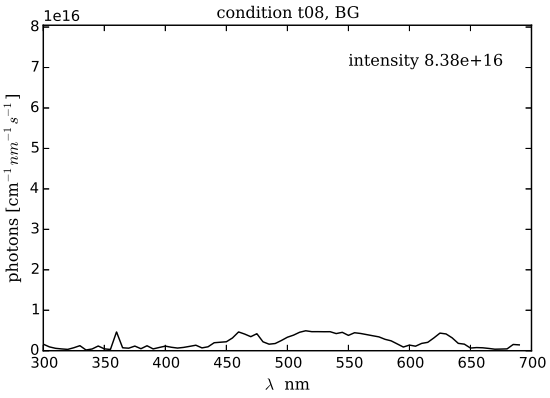


(b) t06 TR left, BG right

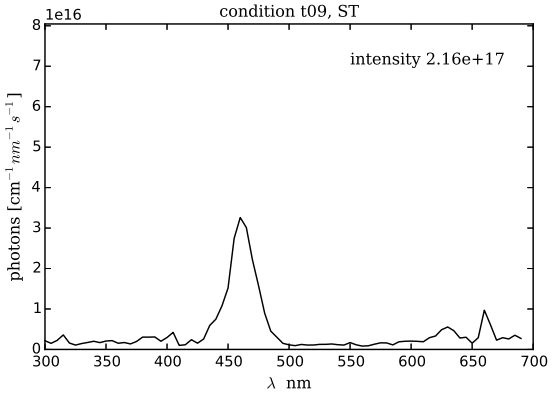


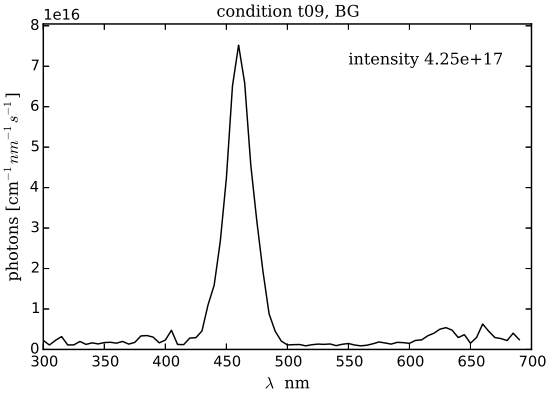
(c) t07 TR left, BG right





(d) t08 TR left, BG right





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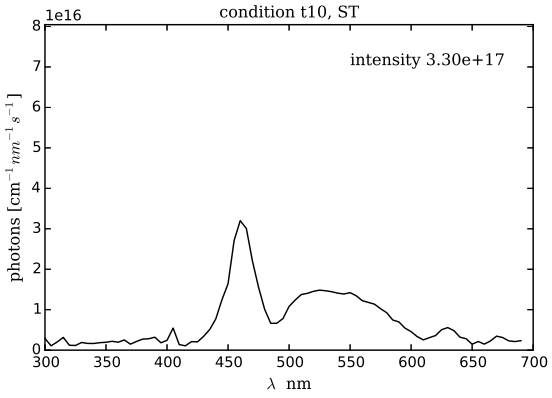
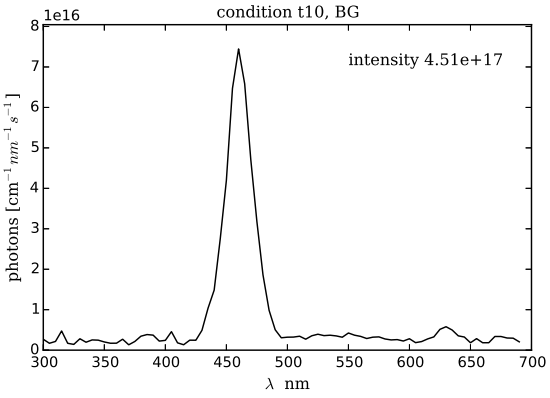
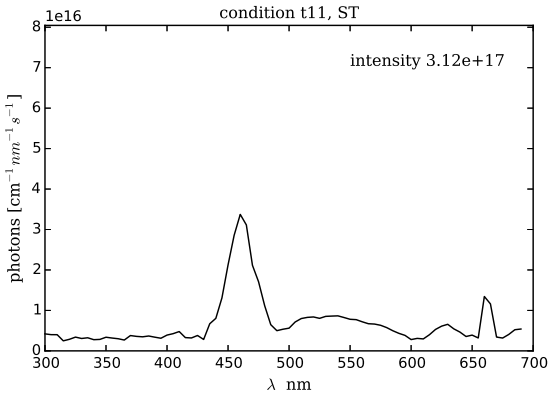
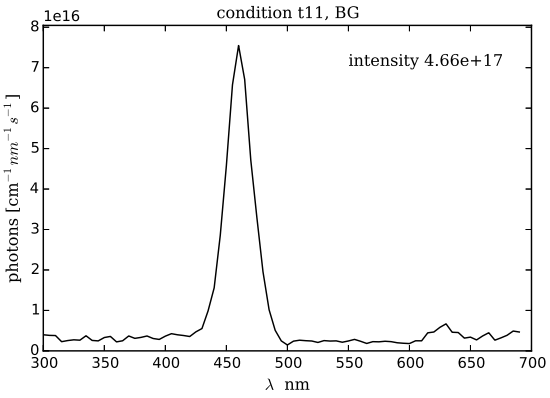


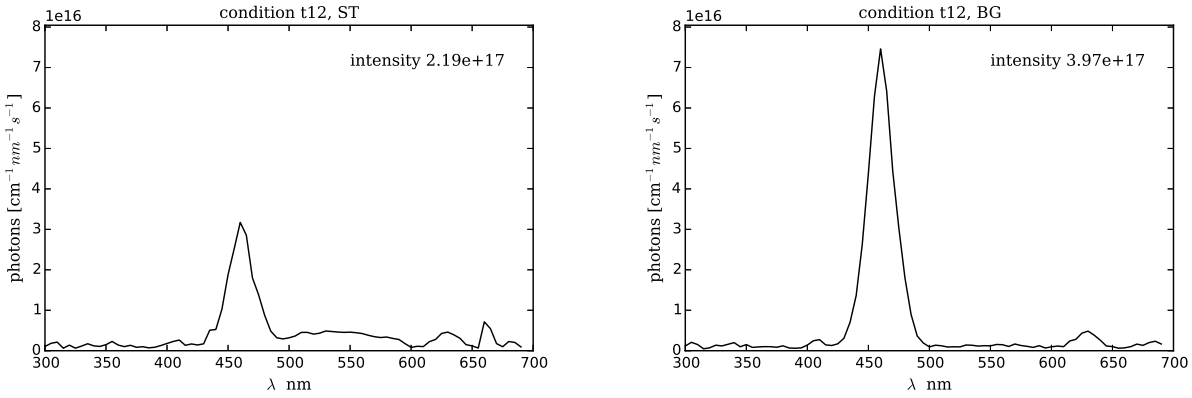


(f) t10 TR left, BG right

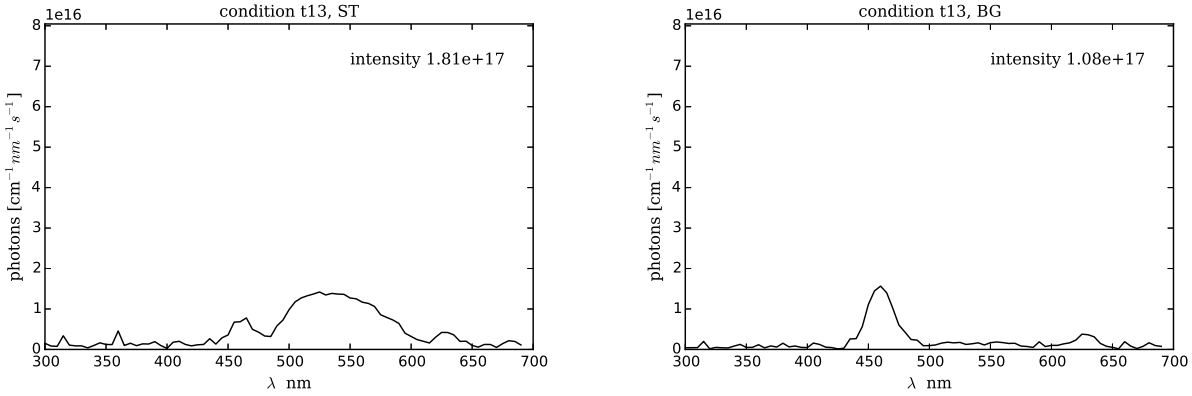




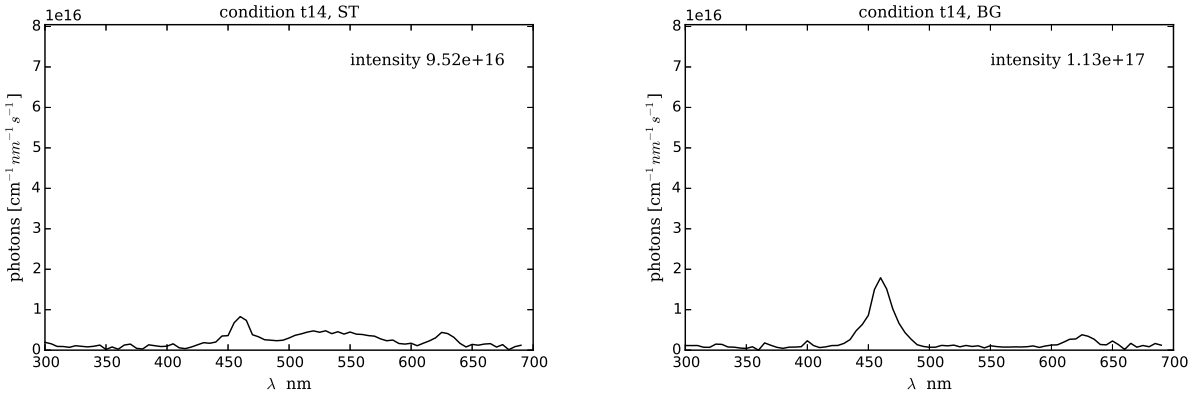
(g) t11 TR left, BG right



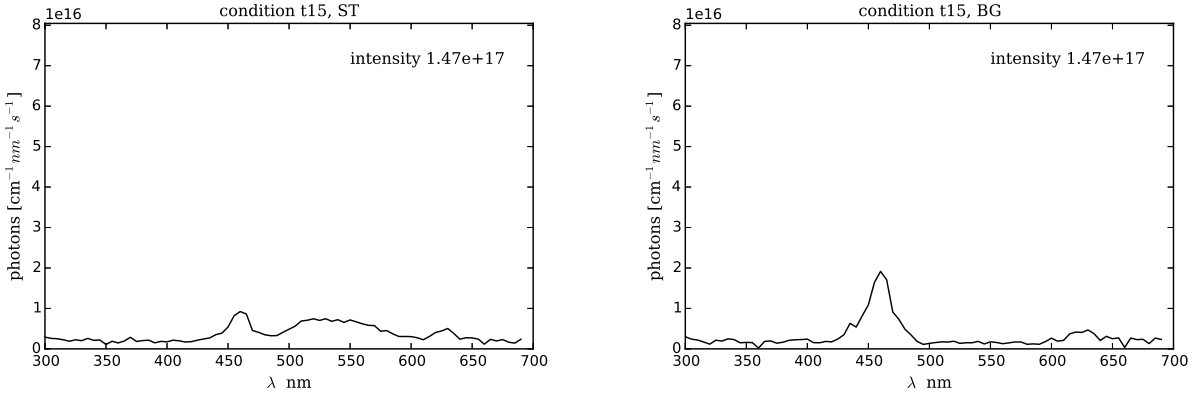
(h) t12 TR left, BG right



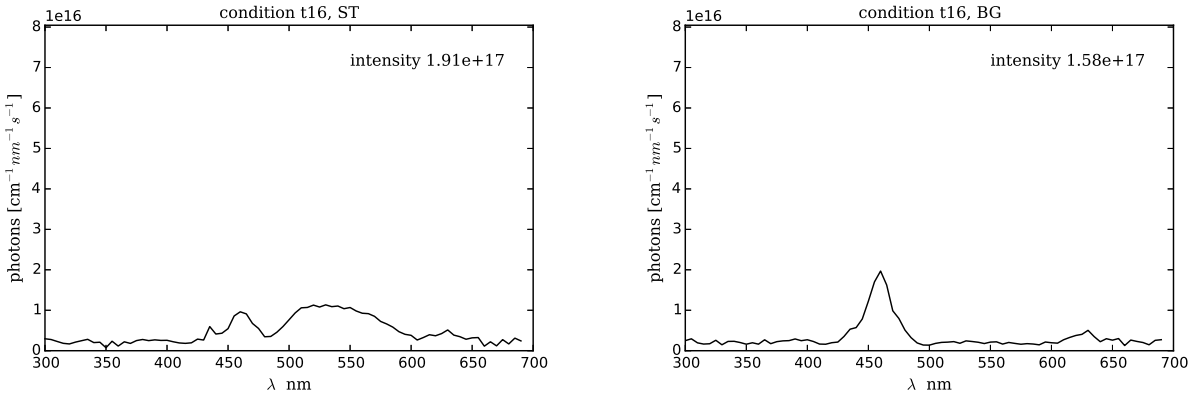
(i) t13 TR left, BG right



(j) t14 TR left, BG right

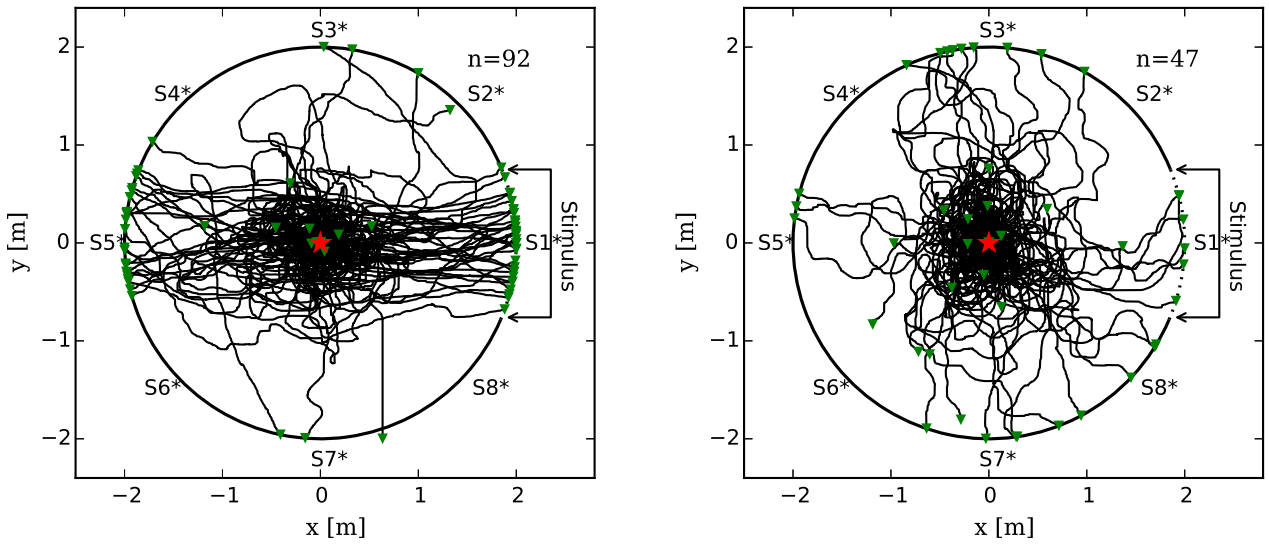


(k) t15 TR left, BG right

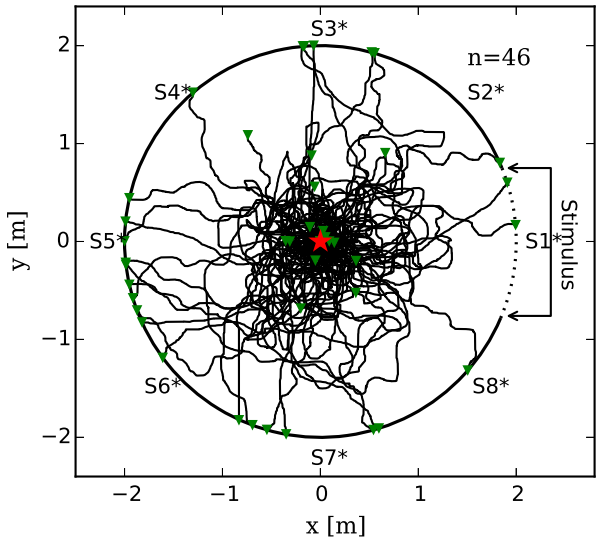


(l) t16 TR left, BG right

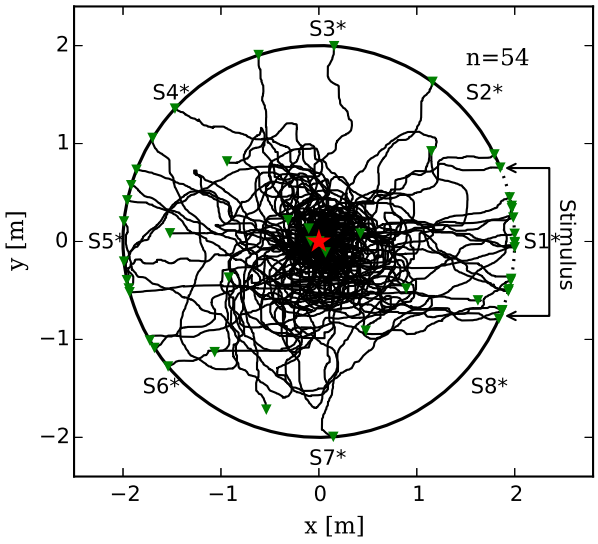
Figure 13: Additional spectra for figure 10 showing the reflected spectra of the target (TR) left and the background (BG) right for experiment II. The colour blue has a much narrower distribution (λ_{max} at 450 nm) than the colour green (λ_{max} at 500 - 550 nm). The peak of the red channel is due to the red rim that had to be projected to prevent the projectors from adjusting the intensity. All conditions are described in table 2.



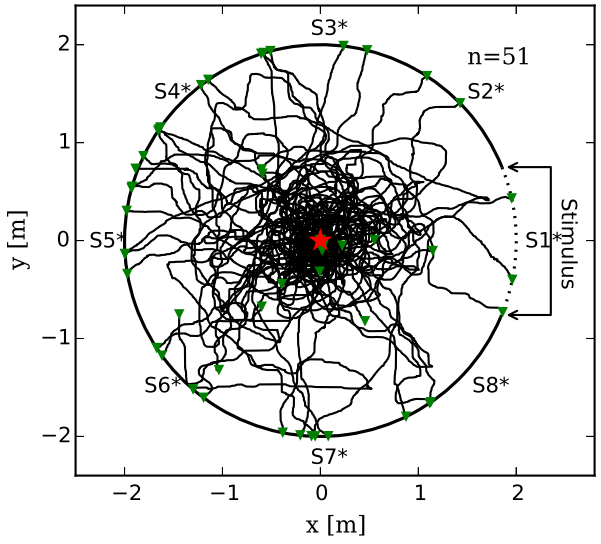
t01 t02



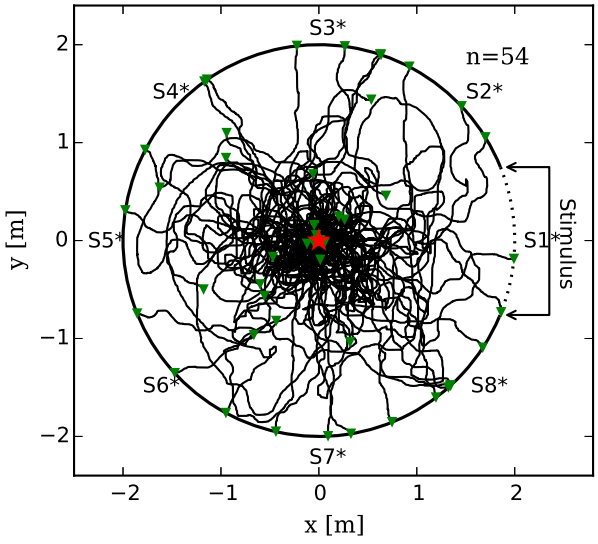
t03



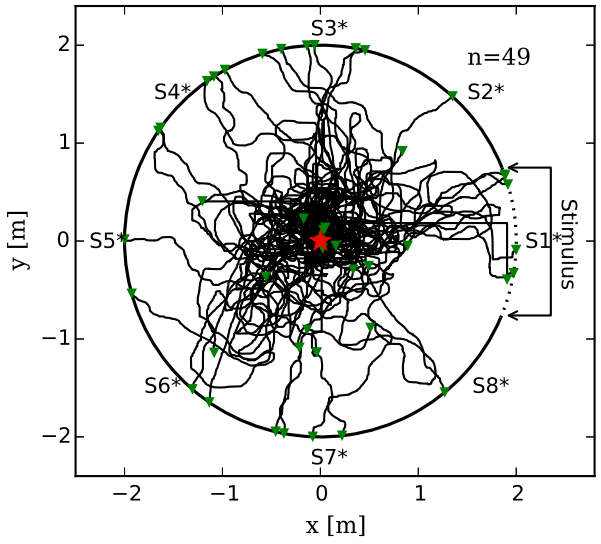
t04



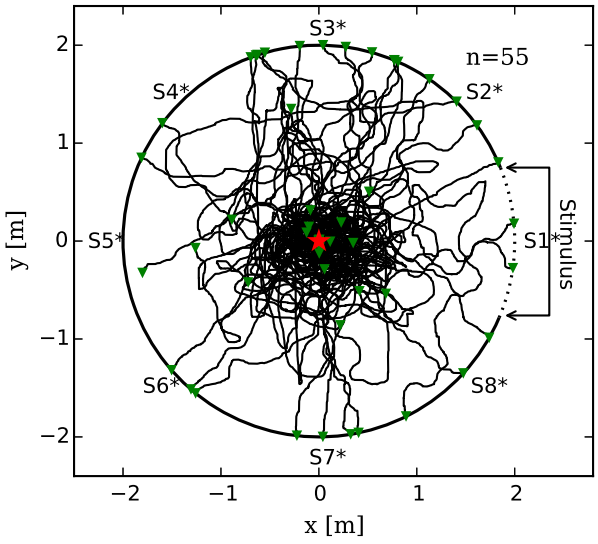
t05



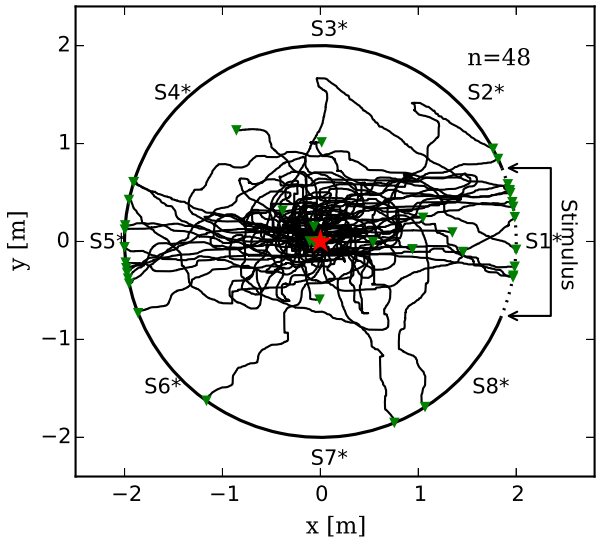
t06



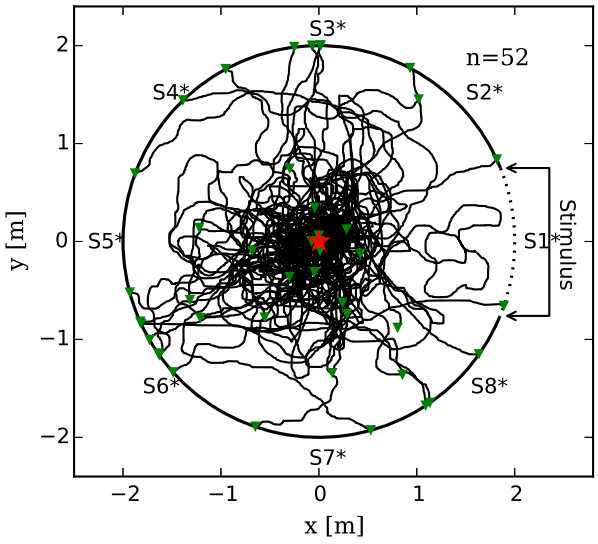
t07



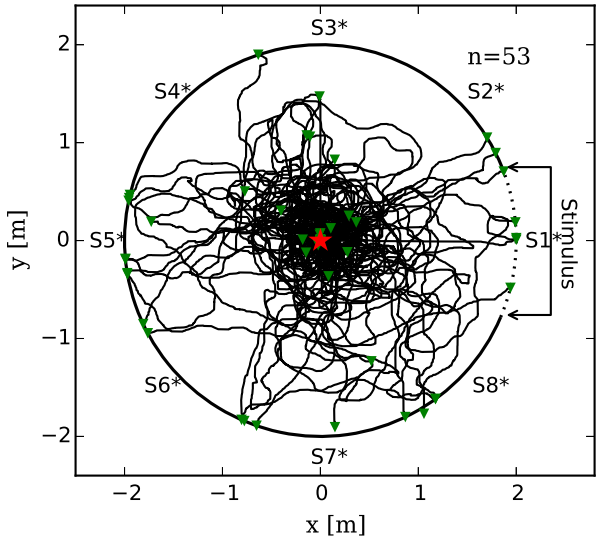
t08



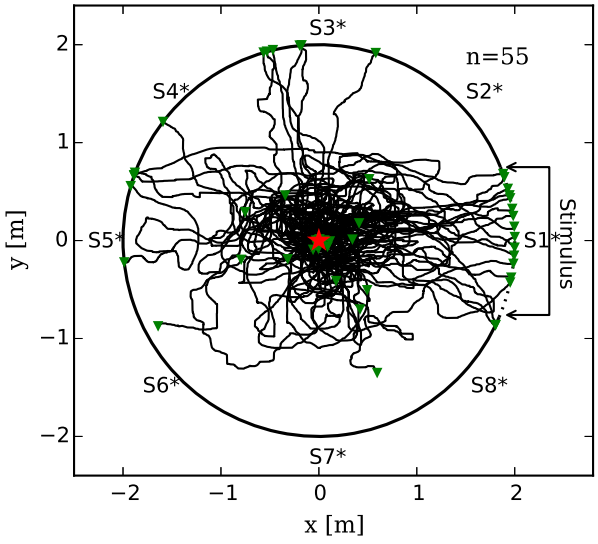
t09



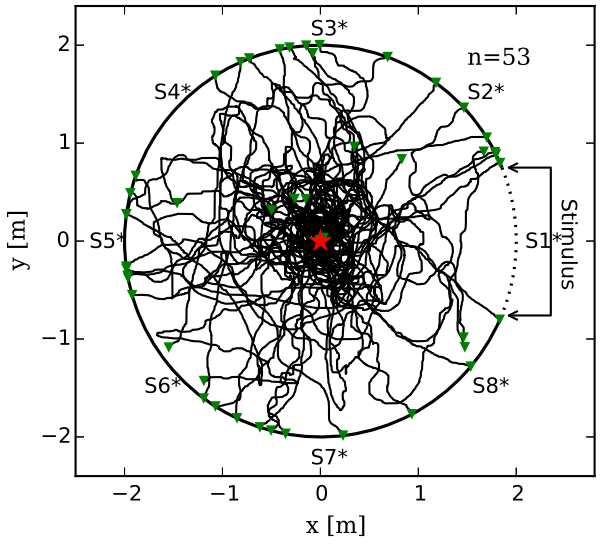
t10



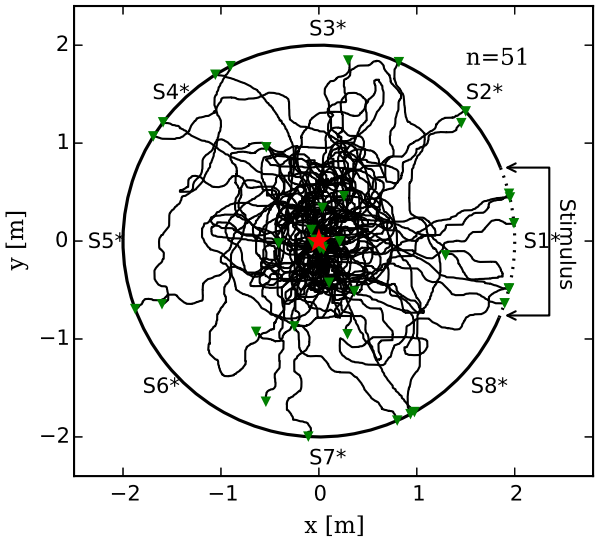
t11



t12



t13



t14

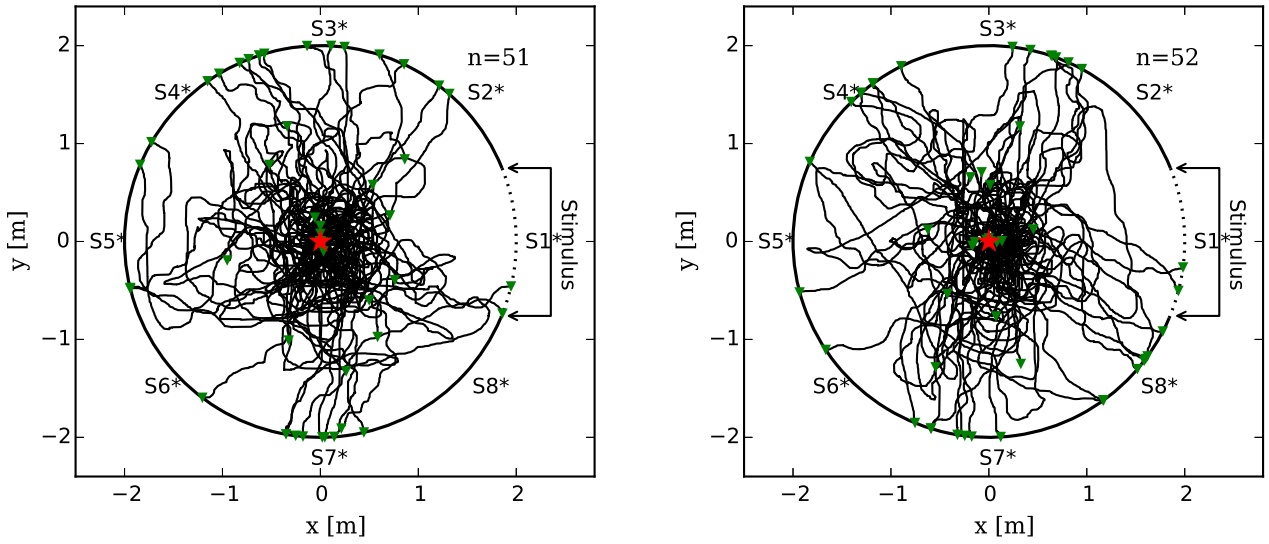


Figure 14: All valid runs that were recorded in experiment II. Trajectories are rotated, so that the target is at 0° which is S1; green triangle marks the endpoint of a run, red star marks the start point (0,0). Spiders showed a significant attraction to the target if it was darker than the background (t01, t04, t09, t12). There was no evidence for a difference whether the target was green or blue.* indicates that the runs were rotated.

CURRICULUM VITAE

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Education

Apr. 2011 – on-going	Master of "Behaviour, Neurobiology and Cognition" at the University of Vienna
Oct. 2006 – Apr. 2011	Bachelor of science (Zoology) at the University of Vienna Bachelor thesis: "Über die Methoden der Präparation von Ultradünnschnitten von T. tubifex und deren Beurteilung unter dem Transmissionenelektronenmikroskop (electron microscopy)"
1996 - 2004	Bundesrealgymnasium Krottenbachstraße, 1190 Wien

Special Courses

WS 2012	Neurobiologie: Visuelle Wahrnehmung Neuronale Schaltkreise im Gehirn von Drosophila
SS 2011	Verhaltensphysiologisches Projektpraktikum
SS 2010	Lernexperimente Submikroskopische Anatomie und Präparationstechnik
SS 2009	Alpenexkursion - zoologisches Projektpraktikum

Publications

Stowers, J. R., Fuhrmann, A., Hofbauer, M., Streinzer, M., Schmid, A., Dickinson, M. H. and Straw, A. D. (2014). Reverse engineering animal vision with virtual reality and genetics. Computer 47(7), 38–45.

Teaching

2014	Animal Physiology (Practical Lab Course) – University of Vienna Department of Neurobiology
2013	Organ Systems (Practical Lab Course) – University of Vienna Department of Neurobiology Molecular and Developmental Neurobiology (Practical Lab Course) – University of Vienna Department of Neurobiology
2012	Basic statistic (Beginners Lab Course) - University of Veterinary Medicine of Vienna
2011	Basic statistic (Beginners Lab Course) - University of Veterinary Medicine of Vienna Epidemiology (Beginners Lab Course) - University of Veterinary Medicine of Vienna