The red flour beetle *Tribolium castaneum* is emerging as a further standard insect model beside *Drosophila*. Its genome is fully sequenced and it is susceptible for genetic manipulations including RNA-interference. We use this beetle to study adult brain development and plasticity primarily with respect to the olfactory system. In the current study, we provide 3D standard brain atlases of freshly eclosed adult female and male beetles (A0). The atlases include eight paired and three unpaired neuropils including antennal lobes (ALs), optic lobe neuropils, mushroom body calyces and pedunculi, and central complex. For each of the two standard brains, we averaged brain areas of 20 individual brains. Additionally, we characterized eight selected olfactory glomeruli from 10 A0 female and male beetles respectively, which we could unequivocally recognize from individual to individual owing to their size and typical position in the ALs. In summary, comparison of the averaged neuropil volumes revealed no sexual dimorphism in any of the reconstructed neuropils in A0 *Tribolium* brains. Both, the female and male 3D standard brain are also used for interspecies comparisons, and, importantly, will serve as future volumetric references after genetical manipulation especially regarding metamorphic development and adult plasticity.

**Keywords:** brain, olfactory system, antennal lobe, insect, neuropil, digital neuroanatomy, coleoptera

**INTRODUCTION**

The red flour beetle *Tribolium castaneum* Herbst, 1797 (Bonneton, 2008), which is a major pest of stored grains, grain products, and other dried food, is emerging as a further standard insect model beside *Drosophila*. Its powerful reverse genetics (systemic RNA-interference; Bucher et al., 2002; Tomoyasu and Denell, 2004; Tomoyasu et al., 2008), the recently published full genomic sequence (Richards et al., 2008) and the established transgenesis systems (Berghammer et al., 1999) transform *Tribolium* into a primary model system. Since recently, an insertional mutagenesis screen database provides mutants and enhancer trap lines for the growing *Tribolium* community (http://134.76.20.145/Default.aspx). Meanwhile, *Tribolium* has become one of the most important models in the field of evolution and development (“evo-devo”) because its development is more “insect typical”, compared to that of the classical system *Drosophila* (Klingler, 2004). Currently, only little information on the brain or its embryonic and metamorphic development is available. With our pioneering study we provide for the first time anatomical descriptions for most of the brain areas of adult *Tribolium* including 3D reconstructions and an average brain atlas for selected brain neuropils. With the latter, we present the first standardization of a coleopteran brain.

Brains are typically organized in defined neuropils, which can usually be characterized by their spatial location, gross anatomy, and often by a certain function. For example, the olfactory bulbs of vertebrates and the antennal lobes (ALs) of insects have been attributed to be the first processing centers for olfactory information (for a review see Hildebrand and Shepherd, 1997). Compared to most vertebrate brains, insect brains are miniature versions being typically comprised of a lower number of neurons and neuropils. Owing to the lower complexity and certain technical advantages, insects have been widely used as models to study principal mechanisms of information processing and integration, in the context of defined sensory inputs but also complex behaviors including learning and memory formation (e.g. Menzel, 2001; Heisenberg, 2003).

Brains of animals of the same or of evolutionary related species typically share the same principal organization. For example in neopteran insects, the central olfactory pathway seems to be well conserved (Strausfeld et al., 1998; Schachtner et al., 2005). However, even within the same species, no brain is identical with the next, differing in size and shape of certain brain neuropils. These individual differences can result from a variety of parameters which are influencing brain organization during development but also during adulthood. In insects, such factors include brood temperature, sex, age, and experience (Groh et al., 2004; Technau, 2007; Molina and O’Donnell, 2008).

To study sexual brain dimorphism or the influence of defined parameters (ranging from single molecules to social experience) on brain development or adult plasticity, average or standardized brains or brain areas are needed to relate individual variations to...
each other. Advances in imaging techniques, 3D reconstruction software, and computer power led so far to 3D reconstructions and subsequent standardization of brain areas of four insect species including *Drosophila melanogaster* (Rein et al., 2002; Jenett et al., 2006), the honeybee *Apis mellifera* (Brandt et al., 2005), the desert locust *Schistocerca gregaria* (Kurylas et al., 2008), and the sphinx moth *Manduca sexta* (el Jundi et al., 2009). To obtain such a standard insect brain, two methods have so far been established: The virtual insect brain (VIB) protocol and the iterative shape averaging (ISA) method. While the VIB protocol was mainly developed to compare wild type and genetically manipulated *Drosophila* (Rein et al., 2002; Jenett et al., 2006), the ISA method, first used for the honeybee, was aimed to register single reconstructed neurons from various individuals into one standard brain (Rohlfing et al., 2004; Brandt et al., 2005). Although the ISA method provides a far better representation of relative locations of brain areas, this high registration quality comes with the tradeoff of missing volumetric consistency for the neuropils. This means, a neuropil label of the standardized ISA brain does not represent the mean volume of all corresponding individual neuropil labels (Kuß et al., 2007; Kurylas et al., 2008). Thus, the VIB script remains advantageous for fast inter- and intraspecific comparisons of neuropils including sex-specific differences, while preserving volumetric consistency.

In the current study, we reconstructed in three dimensions and subsequently standardized brain areas of both sexes of the red flour beetle *Tribolium castaneum* using the VIB protocol. The aims of our study were to (1) compare adult brain neuropil volumes regarding sexual dimorphism (2) provide an adult female and male standard brain at A0 (freshly eclosed adults) as volume references for future genetical and behavioral studies, and (3) to compare the standard volumes of brain areas with previously published standard volumes of homologous brain areas of other neopteran insects. To obtain the desired datasets we labeled whole brains with an antiserum against the synaptic vesicle protein synapsin to visualize neuropil areas, analyzed the staining using confocal laser scanning microscopy, 3D reconstructed the selected brain neuropils using the software AMIRA (Visage Imaging, Fürth, Germany), and subsequently registered and standardized the neuropils using the (VIB) protocol. A standardization using the ISA method can be computed on request. Comparing the standardized neuropil volumes between females and males revealed no obvious sexual dimorphism in A0 *Tribolium* brains.

**MATERIALS AND METHODS**

**ANIMALS**

Wild type *Tribolium castaneum* (San Bernardino; Sokoloff, 1966) stock for egg laying was kept in plastic boxes (20 × 18 × 18 cm) in walk-in environmental chambers at 26°C under constant darkness. The boxes were half filled with substrate containing organic whole wheat flour supplemented with 5% dried yeast powder. To prevent sporozoan infections we added 0.05% (w/w) Fumidil-B (J. Webster Laboratories Inc., Princeville, Kanada; Berghammer et al., 1999). For egg collection, we used similar procedures as described by Huetteroth and Schachtner (2005) and el Jundi et al. (2009). Whole brains were dissected in a drop of cold PBS (phosphate-buffered saline, 0.01 M, pH 7.4) and fixed subsequently in 4% formaldehyde (Roth, Karlsruhe, Germany) in 0.01-M PBS for 1–2 h at room temperature. The brains were then rinsed five times for 10 min at room temperature in 0.01 M PBS followed by preincubation for 1–2 days at 4°C in 5% normal goat serum (NGS; Jackson ImmunoResearch, Westgrove, PA, USA) in 0.01 M PBS containing 0.3% Triton X-100 (PBST). The monoclonal primary antibody from mouse against a fusion protein consisting of a glutathione-S-transferase and the first amino acids of the presynaptic vesicle protein synapsin I coded by its 5’-end (SYNORF1, Klages et al., 1996) was used to selectively label neuropilar areas in the brain (3C11, #151101 (13.12.06), kindly provided by Dr. E. Buchner, Würzburg). Its specificity in *T. castaneum* was shown with Western blot (Utz et al., 2008). The brains were incubated in a 1:100 dilution of the synapsin antibody in PBST containing 2% NGS for 2–3 days at 4°C. Subsequently the brains were rinsed three times for 15 min with PBST before they were incubated with the secondary goat anti-mouse antibody conjugated to Cy5 (1:300, catalog code 115-175-146, lot 71608, Jackson ImmunoResearch, Westgrove, PA, USA) in PBST and 1% NGS for 2 days at 4°C. Afterwards the brains were rinsed again with PBST five times for 10 min and subsequently dehydrated in an ascending ethanol series (50%, 70%, 90%, 95%, and two times 100%, for 2.5 min each) and then cleared in methyl salicylate (Merck, Gernsheim, Germany), until the tissue was transparent. At last the brains were mounted in Permount (Fisher Scientific, Pittsburgh, PA, USA) between two coverslips using two reinforcing rings as spacers (Zweckform, Oberlaimund, Germany) to prevent compression of brains.

**CLSM IMAGE ACQUISITION AND PROCESSING**

The wholemount preparations for the standard brains were scanned with a confocal laser scanning microscope (CLSM, Leica TCS SP2) at 512 × 512 pixel resolution by using a 40× oil immersion objective.
angles served as the symmetry criterion. Both, the results of the
neuropil pairs (AL–Me, AL–Ca, Ca–Me). The sum of the three
scalar product. In this way, we calculated angles between three
the vectors between the centers of the paired neuropils using the
step, we calculated the symmetry of the brain areas by calculat-
calculated for all 20 brains of each sex respectively. In a second
were summed up, and the differences to the mean distance were
distances of the centers of each of the reconstructed neuropils
Kurylas et al. (2008) and el Jundi et al. (2009) we calculated the
neuropils. The selection contained three steps. First, according to
optimal position and symmetry of the reconstructed
position of individual neuropils in the visualized standard brain.
The VIB protocol used for registration and standardization was
registered and standardization protocol was
Diptera (Strausfeld, 2005), and Heteroptera (Settembrini and
sions (8 paired and 3 unpaired neuropils). In the optic lobes,
which we were able to unambiguously delimit in all three dimen-
lobe (vL) and medial lobe (mL), and the calyx (Ca) (Figures 1A-b,
′-lobe, β-lobe, and γ-lobe, which lay very tight together and which typically are one protrusion after reconstruction, the other lobes of the pedunculus, the α- and α’-lobe (Zhao et al., 2008) are discernible protrusions in our
body axis.

The VIB protocol used for registration and standardization was
described in detail by Jenett et al. (2006) and is available at http://
standardized brain. To overcome a subjective bias, we selected the template brains
and three unpaired neuropilar structures in 20 female and 20
male T. castaneum brains provided the underlying matrix of all
computation processes performed (i.e. polygonal surface mod-
forms (8 paired and 3 unpaired neuropils). In the optic lobes,
we reconstructed the medulla (Me), the lobula (Lo), the lobula
plate (LoP), and the accessory medulla (aMe) (Figures 1A-d,
B-c’ and 2). The LoP which lays posterior to the Lo is exclusively
found in Ephemeroptera, Trichoptera, Coleoptera, Lepidoptera,
Diptera (Strausfeld, 2005), and Heteroptera (Settembrini and
Villar, 2005).

In the central brain we divided the mushroom body into two
neuropils, the pedunculus (Pe), which contained the vertical lobe
(VL) and medial lobe (ML), and the calyx (Ca) (Figures 1A-b,
B-a,b’ and 2). Although visible in the synapsin immunostaining,
we refrained from including subunits of the pedunculus described
for the moth Spodoptera littoralis (Sjöholm et al., 2005) or Bombyx
mori (Fukushima and Kanzaki, 2009); the resulting complexity of the
pedunculus would have greatly interfered with standardiza-
tion procedures, and would have also interfered with interspecies
comparison. Nevertheless, with the exception of the β’, and γ-lobe,
which lay very tight together and which typically are one protrusion after reconstruction, the other lobes of the pedunculus, the α-, α’-, and β- lobe (Zhao et al., 2008) are discernible protrusions in our
reconstructions (Figure 2). Between the left and right mushroom
body lies the central complex, which comprises the protocerebral
bridge (PB), the upper and lower unit of the central body (CUB,
CBL) and a small paired neuropil ventrally attached to the central
body, the noduli (No) (Figures 1A-c,e,f,B-b’ and 2). The anterior-
most labeled neuropils were the deutocebral antennal lobes
(AL, Figures 1A-a and 2).

The VIB protocol requires a template brain which defines the
position of individual neuropils in the visualized standardized brain.
To overcome a subjective bias, we selected the template brains
according to optimal position and symmetry of the reconstructed
neuropils. The selection contained three steps. First, according to
Kurylas et al. (2008) and el Jundi et al. (2009) we calculated the
distances of the centers of each of the reconstructed neuropils
to the center of the respective brain. All distances for each brain
were summed up, and the differences to the mean distance were
accomplished for all 20 brains of each sex respectively. In a second
step, we calculated the symmetry of the brain areas by calculat-
ing the difference of the angles between the vectors connecting
the centers of the neuropils. To obtain the angles, we calculated the
vectors between the centers of the paired neuropils using the
scalar product. In this way, we calculated angles between three
neuropil pairs (AL–Me, AL–Ca, Ca–Me). The sum of the three
angles served as the symmetry criterion. Both, the results of the
distance and the angle calculations were normalized, with the
worst brain set to one for each criterion. In the resulting com-
bined ranking, the normalized values of both criteria were added.
The third criterion for the choice of the template brain was a
visual inspection of the three best ranked brains for each sex
respectively. For the male template, we choose the brain ranking
at number one according to the first two criteria. For the female
template we choose number two, because visual inspection of the
three best ranked brains revealed that the left peduncle of female
brain number one was somehow twisted towards the midline. In
the female ranking, brains number one and two were very close
together. The choice of the template brain does not influence the
resulting standard volumes (Kurylas et al., 2008). For creation of
standard brain neuropil labels, we chose an overlap threshold of
40% for all neuropils.

The statistical analysis of these data was obtained using Excel
XP for Windows. The synapsin-immunoreactivity (syn-ir) in
Figures 1 and 5 was auto-contrasted in the OrthoSlice module of
AMIRA. For visualization, neuropils segmented in AMIRA, were
filled with transparent colored labels using Adobe Photoshop 8
(Adobe Systems, San Jose, CA, USA). Snapshots taken in AMIRA
and Pictures edited in Photoshop (Figures 1–3 and 5), and dia-
grams generated with Excel XP (Figures 4 and 6) were imported
to Corel Draw 13 (Corel Corporation, Ottawa, Ontario, CA, USA)
without any further modification.

RESULTS
RECONSTRUCTED NEUROPILS
Of all major areas of the Tribolium brain we reconstructed those
which we were able to unambiguously delimit in all three dimen-
sions (8 paired and 3 unpaired neuropils). In the optic lobes,
we reconstructed the medulla (Me), the lobula (Lo), the lobula
plate (LoP), and the accessory medulla (aMe) (Figures 1A-d,
B-c’ and 2). The LoP which lays posterior to the Lo is exclusively
found in Ephemeroptera, Trichoptera, Coleoptera, Lepidoptera,
Diptera (Strausfeld, 2005), and Heteroptera (Settembrini and
Villar, 2005).

The selected 19 neuropils of the male and female brains were
labeled with the segmentation editor in AMIRA 4.1 (Visage
Imaging, Fürth, Germany). For the eight individual glomeruli of the
right ALs we used AMIRA 5.2.1. For the segmentation and
reconstruction details we refer to Kurylas et al. (2008). In short,
semi-automatically created voxel-based label fields of eight paired
and three unpaired neuropilar structures in 20 female and 20
male T. castaneum brains provided the underlying matrix of all
computation processes performed (i.e. polygonal surface mod-
elns, morphometric analysis, and shape averaging). For orientation
guidance, brain outlines were reconstructed separately. This label
field however was not standardized. The color code for neuropils of
the standard brains is consistent with Brandt et al. (2005), Kurylas
et al. (2008), and el Jundi et al. (2009). We offer the AMIRA label
fields with color codes as online downloadable (Supplement 1). The
orientation of the brain structures is given with respect to the

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Representative outlines of all labels of these selected neuropils are shown in frontal and horizontal slices (Figure 1), an animation of all orthogonal sections of this brain can be found in the supplementary material (Supplement 2). Additionally, all reconstructed neuropils are displayed three-dimensionally to provide a 3D visualization of the whole brain (Figure 2).

**THE STANDARD BRAINS**

To apply the VIB protocol on the 3D brain reconstructions, one brain reconstruction had to be chosen as positional reference (Jenett et al., 2006). To reduce a subjective bias, we selected the template brains according to (1) position and (2) symmetry of the reconstructed neuropils, and (3) final visual inspection of the dorsal to ventral as described in (D). (C) The color code of the labeled neuropils is consistent with Brandt et al. (2005), Kurylas et al. (2008), and el Jundi et al. (2009). AL, antennal lobe; Ca, Calyx; CBL, lower unit of the central body; CBU, upper unit of the central body; aMe, accessory medulla; Lo, lobula; LoP lobula plate; Me, medulla; No, nodulus; PB, protocerebral bridge; and Pe, pedunculus with lobes. (D) Volume rendered (Voltex) view of the template brain from dorsal and frontal. The sections (a–f) and (a′–c′) represent the positions of the optical sections in (A,B). Orientation bars, p, posterior; m, median; d, dorsal. All scale bars, 50 µm.
three best brains resulting from criteria one and two (see Section “Materials and Methods”). The template brain used for generating the male standard brain is shown in Figures 1 and 2. For the female and the male standard brain we reconstructed selected neuropils of 20 individual female and 20 individual male brains of freshly eclosed (A0) *T. castaneum*. With the VIB protocol we generated three-dimensional standard atlases of both sexes consisting of 19 neuropils (eight paired and three unpaired neuropils), including both hemispheres of the brain. The neuropil surface model and the corresponding average intensity map produced by direct volume rendering of the male standard brain are shown in Figures 3A–C,A’–C’ from anterior, ventral and posterior. Volume rendering of all 20 label images after non-rigid registration reveal the high quality of registration (Figures 3D,E). Clear deviations are only visible in the vL of the MBs (Figures 3D,E). An animated view of the male standard brain can be seen in the online supplement (Supplement 3).

**COMPARISON OF THE FEMALE AND MALE BRAINS**

The VIB protocol also generates the standard volumes for each of the reconstructed brain areas of the 20 female and 20 male brains respectively. Table 1 gives mean volumes, standard deviation and standard error of absolute and relative volumes of all 19 areas. Within each sex, a comparison of the volumes of the corresponding left and right brain neuropils using the two-tailed student t-test revealed no significant difference (not shown). Comparing the volumes of corresponding neuropils between females and males resulted also in no significant difference (Figure 4).
Tribolium standard brain

DISCUSSION

Tribolium castaneum belongs to the most species-rich and most diverse order in the animal kingdom; Coleoptera comprise about 40% of all insect species and thus about 30% of all living animal species (Grimaldi and Engel, 2005; Hunt et al., 2007; Hauser et al., 2008). Worldwide, Tribolium is a major pest for stored grain and grain products and serves as a powerful model for the study of...
general insect development and evolution. Owing to the feasibility of transgenic approaches, such as powerful reverse genetics based on systemic RNA-interference, directed gene expression, the recently published full genome sequence, easy culturing, a short life cycle, high fecundity and longevity, Tribolium is emerging as a model system at many fronts. With the current study, we provide a reference for future anatomical studies of the brain in connection with genetic manipulation and external parameters as e.g. odor- or social environment and adaptive learning. The average brain atlas comes from freshly eclosed Tribolium of both sexes. Since we are especially interested in the development and plasticity of the olfactory system, we established an anatomical and volumetric reference of eight selected female and male olfactory glomeruli. Compared to existing insect standard brains, the T. castaneum standard poses - together with the Drosophila standard - the smallest brain.

### STANDARD BRAIN GENERATION

Two methods have been established to obtain a standard insect brain: (1) the VIB protocol, as used for the fruit fly, the desert locust, and the sphinx moth (Rein et al., 2002; Jenett et al., 2006; Kurylas et al., 2008; el Jundi et al., 2009), and (2) the ISA method, as used for the honeybee and also the desert locust (Rohlfing et al., 2004; Brandt et al., 2004).

### Table 1 | Volume measures of neuropil structures in the male and female standard brain of T. castaneum.

Mean volume (Mean vol.), relative volume (Rel. vol.), standard deviation (SD), relative standard deviation (Rel. SD), standard error (SE), and relative standard error (Rel. SE) of all segmented brain areas in the male (n = 20) and female (n = 20) standard brain of T. castaneum.

| Structure                   | Sex | Mean vol. (μm³) | Rel. vol. (%) | SD (μm³) | Rel. SD (%) | SE (μm³) | Rel. SE (%) |
|------------------------------|-----|----------------|---------------|----------|-------------|----------|-------------|
| Medulla (left)              | ♂   | 30339.91       | 14.85         | 7271.10  | 23.97       | 1625.87  | 5.36        |
|                             | ♀   | 34216.66       | 16.34         | 10668.84 | 31.18       | 2325.22  | 6.97        |
| Medulla (right)             | ♂   | 31172.43       | 15.25         | 6053.76  | 19.42       | 1353.66  | 4.34        |
|                             | ♀   | 34157.33       | 16.91         | 9922.73  | 29.05       | 2162.61  | 6.50        |
| Lobula plate (left)         | ♂   | 4824.63        | 2.36          | 1292.49  | 26.79       | 289.01   | 5.99        |
|                             | ♀   | 5251.18        | 2.60          | 850.56   | 16.20       | 185.37   | 3.62        |
| Lobula plate (right)        | ♂   | 4599.73        | 2.25          | 1023.55  | 22.25       | 228.87   | 4.98        |
|                             | ♀   | 5073.72        | 2.51          | 681.34   | 13.43       | 148.50   | 3.00        |
| Inner lobula (left)         | ♂   | 8685.43        | 4.25          | 1828.24  | 21.05       | 408.81   | 4.71        |
|                             | ♀   | 9554.28        | 4.73          | 1735.88  | 18.17       | 378.33   | 4.06        |
| Inner lobula (right)        | ♂   | 9512.70        | 4.66          | 2256.13  | 23.71       | 504.49   | 5.30        |
|                             | ♀   | 9624.48        | 4.77          | 2820.86  | 29.31       | 614.79   | 6.55        |
| Accessory medulla (left)    | ♂   | 1143.63        | 0.56          | 385.77   | 33.73       | 86.26    | 7.54        |
|                             | ♀   | 1173.94        | 0.58          | 350.44   | 29.85       | 76.38    | 6.67        |
| Accessory medulla (right)   | ♂   | 1159.82        | 0.57          | 340.94   | 29.40       | 76.24    | 6.57        |
|                             | ♀   | 1230.22        | 0.61          | 406.31   | 33.03       | 88.55    | 7.39        |
| Calyx (left)                | ♂   | 6003.60        | 2.94          | 1408.59  | 23.46       | 314.97   | 5.25        |
|                             | ♀   | 5515.65        | 2.73          | 1228.05  | 22.26       | 26765    | 4.98        |
| Calyx (right)               | ♂   | 6191.91        | 3.03          | 1084.28  | 1751        | 242.45   | 3.92        |
|                             | ♀   | 5412.37        | 2.68          | 764.70   | 14.13       | 166.66   | 3.16        |
| Pedunculus (left)           | ♂   | 17571.30       | 8.60          | 4577.99  | 26.05       | 1023.67  | 5.83        |
|                             | ♀   | 15796.60       | 7.82          | 3972.44  | 25.15       | 865.77   | 5.62        |
| Pedunculus (right)          | ♂   | 17256.15       | 8.44          | 4794.07  | 27.78       | 1071.99  | 6.21        |
|                             | ♀   | 16057.83       | 7.95          | 36474.71 | 22.09       | 773.14   | 4.94        |
| Central body upper unit     | ♂   | 10263.34       | 5.02          | 3179.91  | 30.98       | 711.05   | 6.93        |
|                             | ♀   | 8668.66        | 4.29          | 1842.36  | 21.25       | 401.53   | 4.75        |
| Central body lower unit     | ♂   | 2838.02        | 1.39          | 1406.16  | 49.55       | 314.43   | 11.08       |
|                             | ♀   | 2364.33        | 1.17          | 713.66   | 30.18       | 155.54   | 6.75        |
| Protocerebral bridge        | ♂   | 3551.02        | 1.74          | 863.97   | 24.33       | 193.19   | 5.44        |
|                             | ♀   | 3310.99        | 1.64          | 630.28   | 19.04       | 1373.7   | 4.26        |
| Nodulus (left)              | ♂   | 371.08         | 0.18          | 115.22   | 31.05       | 25.77    | 6.94        |
|                             | ♀   | 378.12         | 0.19          | 142.66   | 37.53       | 31.90    | 8.39        |
| Nodulus (right)             | ♂   | 380.17         | 0.19          | 70.73    | 19.51       | 15.42    | 4.36        |
|                             | ♀   | 362.49         | 0.18          | 70.73    | 19.51       | 15.42    | 4.36        |
| Antennal lobe (left)        | ♂   | 24373.09       | 11.93         | 5834.27  | 23.94       | 1304.58  | 5.35        |
|                             | ♀   | 22316.68       | 11.05         | 6733.24  | 30.17       | 1467.48  | 6.75        |
| Antennal lobe (right)       | ♂   | 24105.27       | 11.80         | 6511.04  | 2701        | 1455.91  | 6.04        |
|                             | ♀   | 21492.52       | 10.64         | 6132.60  | 28.53       | 1336.57  | 6.38        |
et al., 2005; Kurylas et al., 2008). The VIB protocol was primarily developed to compare brain areas e.g. between wildtype and genetically manipulated Drosophila, while the ISA method aims to generate a synthetic but realistic standard brain, into which single reconstructed neurons from various individuals could be mapped. The VIB script keeps neuropil volumes rather unchanged, while the ISA method, in contrast, averages anatomical differences on the cost of volume accuracy (Kuß et al., 2007; Kurylas et al., 2008; el Jundi et al., 2009). Both methods require an initial reference or template brain for alignment. While the visualization of the standardized brain areas using the VIB protocol is clearly biased towards this template, the ISA method is thought to be independent of the choice of the template (Guimond et al., 2000; Brandt et al., 2005), with the notable exceptions of orientation and scale. During affine registration in the ISA method, all brains are resized using anisotropic scaling to match the size of the template brain. Thus, the resulting standard volumes of the brain areas generated by the ISA method depend on the choice of the template brain (Rohlfing et al., 2001; el Jundi et al., 2009). Therefore we decided to use the VIB protocol for standardization, since we primarily wanted to compare volumes of neuropils and did not aim for registration of reconstructed neurons (Ro et al., 2007; Kurylas et al., 2008). Given that both methods are established in our lab, a female and a male standard brain calculated by the ISA method could be computed on request.

It has to be noted that our whole mount specimens, like all immunohistochemical preparations, are subjected to considerable tissue shrinking (Bucher et al., 2000; Ott, 2008). Therefore absolute sizes are probably underestimated and make most sense in relative comparisons, i.e. comparisons might only be useful between brains after similar histological treatment. In a previous work, we already showed the usability of 3D reconstructions to quantify adult plasticity in the male antennal lobe of the sphinx moth (Huetteroth and Schachtner, 2005). Since we carefully checked for animal age, the female and male standard brain will serve as a reference in future quantitative studies using genetical or behavioral approaches.

**BRAIN NEUROPIV COMPARISON BETWEEN SEXES**

We found no volumetric differences between females and males in any one of the standardized brain neuropils, including the eight olfactory glomeruli. Anatomical sexual dimorphism in brain structures has been described in a variety of insects primarily with respect to the ALs (for a review see Schachtner et al., 2005). Enlarged glomeruli at the entrance site of the antennal nerve are described for males, as example for cockroaches (Jawlowski, 1948; Neder, 1959; Boeckh et al., 1987), bees (Arnold et al., 1984; Brockmann and Brückner, 2001), ants (Kleineidam et al., 2005; Nishikawa et al., 2008), flies (Kondoh et al., 2003), and moths (reviewed in Anton and Homberg, 1999; Hansson and Anton, 2000). These glomeruli are usually called macroglomeruli or macrogglomerular complex (MGC). These glomeruli appear to be involved in pheromone signal processing (reviewed e.g. in Hansson and Christensen, 1999).

Why did we see no sexual dimorphism in the examined brain areas? In principal we expect sexual dimorphism on the level of the brain areas due to sexual specific input (e.g. in the case of the olfactory system a higher number of receptor neurons responsible for the detection of the female pheromone) and/or due to sexual specific behaviors which have to be coordinated from female or male brains in the context of sexual reproduction. The question is whether this dimorphism can be seen on the level of gross brain anatomy like in the case of the sexual specific glomeruli or whether it is due to a few special neurons or and/or different neurochemistry with only little or no effect on gross morphology. As individual variations in neuropil volumes range in the mean at around 20%, we cannot detect anatomical sexual dimorphism smaller than that. Furthermore, we looked at freshly eclosed animals. Thus, the animals are not sexually mature at that time and the brain has just started to get acquainted to the environmental cues including odor information like e.g. pheromones. Currently, we produce a female and male standard brain atlas for 7-day-old animals to examine how brain anatomy is changing in females and males. In M. sexta for example, the sexual dimorphic male glomeruli increase about 40% in volume during the first 4 days of adulthood (Huetteroth and Schachtner, 2005).

**INTERSPECIES BRAIN COMPARISON**

The relative size of a defined brain area is closely related to its apparent importance for the respective animal (e.g. Barton et al., 1995; Gronenberg and Hölldobler, 1999; Schoenemann, 2006). For example in insects larger optic lobes primarily correlate with larger complex eyes containing more photoreceptor cells, while the volume of ALs correlates with the amount of olfactory sensory axons entering this structure. Likewise, the volumes and the organization of higher order integration centers like the mushroom bodies correlate with the complexity of multimodal sensory integration (e.g. Techau, 2007; Molina and O’Donnell, 2008). Additionally, studies in several insect species demonstrated a correlation of volumes of brain areas with age, caste, sex and experience, including primary sensory integration centers like OL and AL, and higher integration centers like the mushroom bodies (Heisenberg et al., 1995; Barth...
So far, the few published insect standard brains give only a limited view on the respective relative volumes of defined brain areas of these species because they provide (1) data for only one sex (with the exception of *M. sexta*) and (2) one age (*D. melanogaster*: 5-day-old adult females; *Rein et al.*, 2002; *M. sexta*: freshly eclosed adult females and males; *el Jundi et al.*, 2009), or (3) a mixture of different ages (*A. mellifera* foragers: *Brandt et al.*, 2005; *S. gregaria* males: *Kurylas et al.*, 2008). Caste or possible experience dependent differences have also not been taken into account. Thus, a comparison between the relative volumes of the available standardized brain areas has to be judged under these prerequisites (Table 2).

In *T. castaneum*, the optic lobes show the smallest relative volumes, which corresponds to the relative small compound eyes (with 80–83 ommatidia per eye; *Friedrich et al.*, 1996) compared to the other insect species. The ALs in contrast display the largest relative volume, which suggests that *Tribolium* may primarily rely upon chemical cues, a fact which has been generally proposed for insects inhabiting grain storage areas (Levinson and Levinson, 1997; *Barth et al.*, 1997; *Sigg et al.*, 1997; *Julian and Gronenberg*, 2002; *Groh et al.*, 2006; *Technau*, 2007; *Krofczik et al.*, 2008; *Molina and O’Donnell*, 2008; *Maleszka et al.*, 2009; *Snell-Rood et al.*, 2009).
Mushroom bodies (MBs) are generally associated with higher integration processes and learning (e.g., Menzel, 2001; Heisenberg, 2003), but might also serve a general function in the control of behavior (e.g., Huber, 1955a, b; Erber et al., 1987; Zars, 2000; Strausfeld et al., 2009). While the social honeybee far exhibits the largest relative MB volumes, interestingly, Tribolium is second before Drosophila (Table 2). A recent study found similar development of MBs in Tribolium and Drosophila, with the remarkable difference, that adult neurogenesis occurs in Tribolium (Zhao et al., 2008). MBs vary in relative size in different nymphid butterflies, without a correlation with optic or antennal lobe size. Heliconius charitonius for example has almost four times bigger mushroom bodies than other butterflies of that family (Sivinsky, 1989). This is attributed to its relative long life combined with its occurrence in forest habitats with only scattered food resources, and a shared resting place with conspecifics. As discussed by the author, remembering a common resting place and good food sites might be a higher evolutionary constraint for learning ability than finding proper egg-laying sites, which does not necessarily involve memory tasks (Sivinsky, 1989). For the ant Cataglyphis, Wehner et al. (2007) discussed social interaction rather than food gathering for being responsible of bigger mushroom bodies compared to other ant species, an idea originally brought up by von Alten (1910). The relative large size of the MBs in Tribolium suggests a high integrative capacity which may include olfactory components (see above). Additionally, a life expectation of months to years and a long reproductive period (Dawson, 1977) might also justify an investment into a brain structure dedicated to higher integration, memory, and behavioral control.

The function of the central complex still remains elusive, but is probably best described as a central coordinating function in sensory and motor integration (for reviews see Strauss, 2002; Wessnitzer and Webb, 2006; Homberg, 2008). Regarding the relative volume of the central complex, the sum of relative ellipsoid body volume and fan-shaped body volume in the fly and the relative volume of upper and lower units in locust, honey bee, moth, and beetle, Tribolium exceeds even that of the fly. This suggests a more complex function than in all other examined insects. In this context, it would be interesting to have comparable standardized central complex volumes of other coleopterans with different lifestyles e.g. water beetles or non-flying beetles.

Table 2 | Comparison of relative neuropilar volumes between different insect species obtained from five different insect orders, namely Diptera (Drosophila melanogaster: Rein et al., 2002), Hymenoptera (Apis mellifera: Brandt et al., 2005), Orthoptera (Schistocerca gregaria: Kurylas et al., 2008), Lepidoptera (M. sexta, el Jundi et al., 2009), and Coleoptera (T. castaneum, this work). We included the sex and the number of individuals which were used for respective standardization. Only neuropils which have complements in all examined animals were compared (optic lobes: medulla, lobula complex, and lobula plate; antennal lobes, mushroom bodies including calyces and pedunculi, upper and lower unit of the central body).

| Order       | Species                        | Sex | D. melanogaster | A. mellifera | S. gregaria | M. sexta | T. castaneum |
|-------------|--------------------------------|-----|-----------------|--------------|-------------|-----------|--------------|
|             |                                |♀    |♂               |♀            |♂           |♀         |♂            |
| Number of individuals |                                |28   |20              |10           |12          |12         |20           |
| Optic lobes (%)     |                                |79.65|57.91           |72.67        |79.36       |77.35      |50.06        |
| Antennal lobes (%)  |                                |9.36 |8.53            |9.68         |12.86       |15.09      |22.41        |
| Central body (%)    |                                |3.43 |0.91            |1.67         |0.91        |0.89       |5.64         |
| Mushroom bodies (%) |                                |7.56 |32.65           |15.98        |6.87        |6.76       |21.88        |
With the current study we provide a standard female and male brain of freshly closed Tribolium castaneum. These standard brains will serve as a useful tool to study brain development and brain plasticity.

ACKNOWLEDGMENTS
The authors thank Dr. Erich Buchner (University of Würzburg, Germany) for kindly providing the anti-Synapsin antibody and Drs. Jochen Trauner and Gregor Bucher for supplying us with starter colonies of Tribolium. The authors are also grateful to Drs. Uwe Homberg, Ernst Wimmer and Stefan Schütz for many fruitful discussions and Martina Kern and Silke Redelfs for expert technical assistance. This work was supported by a DFG grant (SCHA 678/13-1) to Joachim Schachtner.

SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at http://www.frontiersin.org/systemsneuroscience/paper/10.3389/neuro.06/003.2010/

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

Received: 05 September 2009; paper pending published: 28 November 2009; accepted: 18 January 2010; published online: 03 March 2010.

Citation: Dreyer D, Vitt H, Dippel S, Goetz B, el Jundi B, Kollmann M, Huetteroth W and Schachtner J (2010) 3D standard brain of the red flour beetle Tribolium castaneum: a tool to study metamorphic development and adult plasticity. Front. Syst. Neurosci. 4:3. doi: 10.3389/neuro.06.003.2010

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