

Tiny Eggheads

Brain and body size relationship in the parasitic wasp
Trichogramma evanescens



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Abstract

Studies of allometry in brain size are commonly used to investigate the effects of body size on brain functions and animal intelligence. The general rule is that large animals have relatively smaller brains than small ones (*i.e.* negative allometry). In a double logarithmic plot of brain size versus body size, the relationship between brain and body size is described by the slope of a straight line, the scaling coefficient b ($b < 1$: negative allometry; $b = 1$: isometry; $b > 1$: positive allometry). Among the smallest insects on Earth are parasitic *Trichogramma evanescens* wasps that develop inside host eggs of a variety of butterfly and moth species. Despite their small size (with a body length of approximately 0.3 to 0.7 mm long) they function as many larger animals in their motoric and cognitive abilities and have even been shown to be capable of learning. *Trichogramma evanescens* wasps have heads that are rather large relatively to their body size, suggesting a large investment in brain development and maintenance. The large investment suggests that their brains cannot reduce in size without compromising brain performance, consequently preventing the wasps' body size to reduce even further. This study aimed to investigate the limits of brain size reduction in some of the smallest insects on Earth by finding out what the relationship is between brain size and body size in *Trichogramma evanescens*, and in what way these relationships are influenced by the wasps' genotypes and host species. If *T. evanescens* brains are as small as they could possibly get, small wasps will have brains that are relatively much larger than those of the large wasps. Therefore a very strong negative allometric relationship is expected, with a scaling coefficient b even smaller than the 0.2 – 0.4 range observed in ants.

Three different iso-female strains (representing three wasp genotypes) were reared in a large phenotypic variety of body size, depending on the size of, and number of wasps developing inside, host eggs of two different moth species. The wasps' brain and body volume was measured using confocal laser scanning microscopy and digital three dimensional reconstructions. Regression models allowed the formulation of allometric relationships between brain volume and respectively body volume, hind tibia length, body length, head width and head volume. These analyses reveal that the relationship between brain volume and body volume is described by weak negative allometry, approaching an isometric relationship with a scaling coefficient b of 0.91, meaning that larger wasps have almost similar relative brain sizes as small wasps have. Host species and genotype did not significantly affect this relationship. *Trichogramma evanescens* wasps have brain volumes constituting on average 13% of their total body volume, which is a rather large relative brain size. Both intraspecifically and within the three iso-female strains, absolute brain volume was found to vary by an order of magnitude of up to 5, solely dependent on the amount of

nutrition available during development inside a host egg. The allometric scaling coefficient in *T. evanescens* is very different from the intraspecific coefficients found for ants and vertebrates ($b=0.2 - 0.5$), thereby suggesting that *T. evanescens* wasps apply a very different brain scaling strategy compared to larger animals. Small *T. evanescens* wasps have brains that are relatively only slightly larger than those of large wasps, possibly because a brain volume of about 13% of body volume is the best solution for the wasps and might be fixed in their genotypes. It is possible that the wasps are able to reach their minute brain and body sizes because they could reduce the size of their neurons beyond the minimum of other insect species' neurons. Future investigations should focus on finding out if this brain scaling strategy is applied by all species of very small animals or if it is unique among these minute egg parasitoids.

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Introduction

Humans are generally seen as the most intelligent and behaviourally complex animals that ever occupied this planet. With brains that are very dense and contain more cortical neurons than those of larger brained animals as whales and elephants (Roth and Dicke, 2005), humans are capable of complex cognition that enabled them to manipulate their surroundings and control the Earth.

Apart from the number of neurons in the cortex, the size of the brain is also often said to be important in determining cognitive abilities. However, it is not the absolute brain size that matters. Large animals generally have large brains, because more neurons are required to control a large body. Having a larger brain, however, implies having more and longer neurons to cross larger distances. These neurons subsequently need to be thicker to maintain the same conduction speed and due to their increased numbers, they cannot always keep the same proportion of connections among themselves and target areas (Kaas, 2000). As a consequence, brains that are large in absolute size (e.g. those of elephants and whales) do not necessarily perform better than small ones.

Among large mammals, humans have the largest brains relatively to their body mass (Roth and Dicke, 2005), correlating with cognitive abilities unique in the animal kingdom. This association is also found in other animals. Relatively large brained corvid birds, for example, can use and create tools, show extensive food storage memory and learn by observing other birds. These behaviours point to an ability of causal reasoning, imagination, prospection and skills to act on information flexibility, which are features often used to measure animal intelligence. Chimpanzees show equal complex cognitive abilities, and have the same relative brain size as corvids (Emery and Clayton, 2004).

Throughout the animal kingdom, a clear trend is visible in relative brain sizes: large animals have relatively smaller brains than small ones (Chittka and Niven, 2009; Kaas, 2000; Warton et al., 2006). This tendency is known as Haller's rule (Rensch, 1956) and can be described by a power function, $[\text{brain weight}] = a * [\text{body weight}]^b$. After logarithmic transformation, this results in the linear equation $\log[\text{brain weight}] = b * \log[\text{body weight}] + \log[a]$, in which the scaling coefficient b represents the slope of the line. For Haller's rule to apply, b has to be smaller than 1. In that case, the brain/body size relationship is negative allometric. When the scaling coefficient b is equal to 1, the relationship is isometric. Studies of allometry are used to compare the relative brain sizes between species (interspecific allometry), but also among individuals of the same species (intraspecific allometry). Interspecific relationships focus on brain size differences independently of body size among species, thereby linking relative brain size to a species' specialisations in brain functions and behaviour (Kruska, 2005). For interspecific relationships in

mammals, the allometric scaling coefficient b is usually found to be around 0.75 (Harvey and Krebs, 1990; Isler et al., 2008; Pagel and Harvey, 1989; Wehner et al., 2007), but significantly lower values (all around 0.5) are found for birds, reptiles and ants (Kalisińska, 2005; Wehner et al., 2007).

Intraspecific relationships allow comparisons between populations or races of the same species, but can also be used to study the effects of environmental factors on relative brain size (Kruska, 2005). To determine these relationships a large variation in body sizes within species is required, which is often complicated to realize. In mammals, these studies are therefore often performed by comparing domesticated animals to their wild conspecifics or by comparing different races of domesticated animals to each other (Kruska, 1996; Stuermer et al., 2003). Generally, both in vertebrates and in insects, intraspecific coefficients are about half the size of the interspecific coefficients, ranging from 0.2 - 0.5 (Gonda et al., 2011; Isler et al., 2008; Kruska, 1996; Kruska, 2005; Lopicque, 1907; Riveros and Gronenberg, 2010; Seid et al., 2011; Stuermer et al., 2003; Wehner et al., 2007; Weston and Lister, 2009).

Analyses of allometry in brain size are a necessary step in studying the effects of body size on animal cognition. The downside is that these analyses never rule out genetic influences on the relationships, since even in intraspecific studies genetically different individuals are compared. A third type of allometric relationship, one solely based on genetically identical individuals, would be convenient for studying the phenotypic effects of body size on brain size. Such a relationship would then allow further research on the phenotypic effects of body size on brain functions and intelligence. Unfortunately, these relationships have not yet been made. They require genetically identical animals with a large variability in body sizes, preferably able to show some features of intelligence, such as an ability to learn and store memory. Parasitic wasps of the species *Trichogramma evanescens* fit this description very well.

Trichogramma evanescens is a species of parasitic wasps that are among the smallest insects on Earth, with body lengths that can go under 0.5

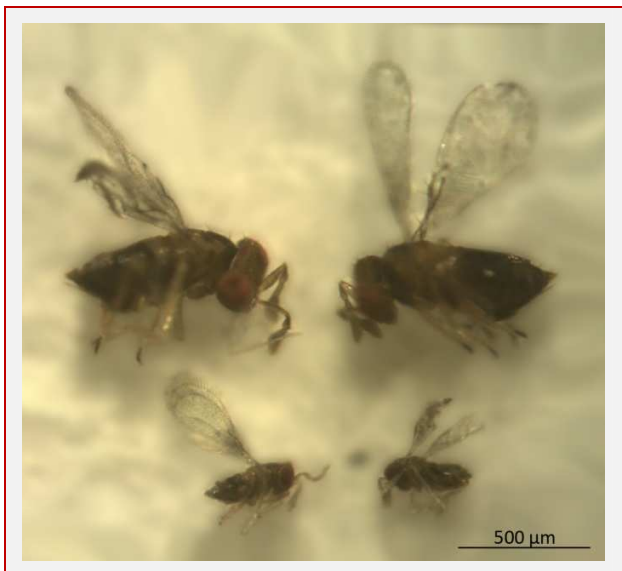


Fig. 1. Example of phenotypic body size variation in *Trichogramma evanescens* wasps. The wasps shown in this picture are females of equal age and belong to the same iso-female strain, yet show an extreme difference in body size.

mm. Being (facultative) gregarious egg parasitoids, the wasps develop inside the eggs of butterflies and moths and can share these eggs with several other wasp larvae. The eventual body size of the wasps depends on the amount of nutrition that was available during their development (Consoli and Parra, 2010; Wajnberg and Hassan, 1994). This means that a wasp that was reared in an unshared, large butterfly egg will be relatively large, whereas a wasp developing in a small egg shared with some siblings will turn out to be relatively small. This difference in body length between small and large wasps can be up to almost three times (Figure 1). Another convenience is that these wasps can be cultured as iso-female strains, in which all wasps descend from one female wasp. The wasps have a haplodiploid mode of sex determination in which haploid males develop from unfertilized eggs whereas diploid females arise from fertilized eggs. After several generations of inbreeding (haplodiploid organisms do not suffer as much from inbreeding as diploids do), the wasps of a strain can be assumed to be genetically identical. The large variability in body sizes would then be purely dependent on the wasps' nutrition during development.

Despite their small size, *T. evanescens* wasps function as many larger animals: they walk, fly, see, smell and show both innate behaviour and even a capability of learning (Dutton and Bigler, 1995; Fatouros et al., 2005; Huigens et al., 2009; Huigens et al., 2010; Pompanon et al., 1997). These rather complex behaviours require brain functions which fit in a minuscule brain of only a few hundred micrometers in length. Although their brains are incredibly small in absolute sense, the wasps' heads are relatively large when compared to their body size (Schmidt and Smith, 1986). A *Trichogramma evanescens* head width is about half the length of the wasp's body, suggesting that they spend a relatively high proportion of their energy in developing and maintaining their brains.

Because the wasps' minute brain is relatively quite large, it is possible that the size of the brain cannot reduce further without compromising brain performance. A smaller brain can be caused by either a reduction in the size of neurons, or in number of neurons, or both (Kaas, 2000). These reductions are limited, however, since a cell body needs to have sufficient space for its nucleus and other cell contents (about 2 μm ; Beutel et al., 2005), axons need to be thick enough to conduct information properly (about 0.1 μm ; Faisal et al., 2005), and a minimum amount of neurons is required for an accurate function of neural circuits (Kaas, 2000).

When these limits are reached in small *T. evanescens* wasps, absolute brain size has attained its minimum. If the brains of the wasps are indeed on the edge of what is physiologically possible, and their brain size cannot be reduced any further without losing vital functions, small wasps should have brains

that are relatively much larger than those of large wasps. When put in terms of allometry, I expect a strong negative allometric relationship between body size and brain size in *Trichogramma evanescens*.

The objective of my MSc thesis is to investigate the relationship between brain size and body size in this minute intelligent insect species, *Trichogramma evanescens*, and in what way this relationship is influenced by the wasps' genotypes and host species. These studies will provide insight into the limits of brain size reduction in the smallest insects on Earth and allow future research on the effects of brain size on insect intelligence, behaviour and neurology.

More specifically, I would like to address the following research questions:

1. Do small wasps have relatively much larger brains than larger wasps?

As stated before, *Trichogramma evanescens* wasps have relatively large heads in which a fully functional brain is present. Their large brain:body size ratio could mean that their cognitive functions are too complex and require too much brain tissue to fit in a smaller brain. If *T. evanescens* brains are as small as they could possibly get, small wasps will have brains that are relatively much larger than those of the large wasps. I therefore expect a very strong negative allometric relationship, with a scaling coefficient b smaller than the 0.2 – 0.4 range observed in ants by Wehner et al. (2007), in which body size has hardly any influence on the size of a wasp's brain. Since females need to associate odours to ovipositing opportunities, they need a fully functional brain adapted to these complex cognitive processes more than males might do. I therefore expect this strong selection on brain volume specifically in female *T. evanescens* wasps, which would reflect in strong negative allometry, and will analyse only females for this reason.

2. Are there any genotypic effects on brain allometry in the wasps?

To test if the relationships I find between body and brain size apply to the entire species of *Trichogramma evanescens*, while at the same time being able to quantify the phenotypic variability that occurs within a specific genotype, I will use three different iso-female strains in my experiments. I assume equal costs and benefits associated to a specific amount of brain tissue for all individuals of the same species, and that all *T. evanescens* wasps therefore show the same relationship between brain size

and body size. Consequently, I expect to find no differences in this relationship between the three iso-female strains.

3. Are there any effects of the host species on brain allometry in the wasps?

If the host species *Trichogramma evanescens* would influence the wasps' relationships between body and brain size, wasps reared using eggs of a specific host could only be compared to wasps that developed inside the eggs of the same host species. Since size variability in *T. evanescens* wasps strongly depends on the size variation of their hosts' eggs, the only way to create a large range of wasp body sizes is by allowing them to parasitize eggs of different host species. It is essential in the comparison of wasps that were bred in eggs of different butterfly species, that the host species does not influence the relationship between the wasps' body and brain size. I assume that the nutrition of a small *Ephestia kuehniella* host egg is converted into wasp body and brain tissue in the same relative proportions as the nutrition of a larger *Mamestra brassicae* host egg. Therefore, I expect the relationships between brain size and body size to be the same in wasps emerging from eggs of *E. kuehniella* as in wasps developing in *M. brassicae* eggs.

Methods

Wasp strains

The wasps used in this thesis were female *Trichogramma evanescens* Westwood wasps (Hymenoptera: Trichogrammatidae) of three iso-female strains (GD011, GD025 and GD034). The three females that were used to create these strains were collected in 2006 from a cabbage field located in the city of Wageningen (the Netherlands). Their offspring has been allowed to inbreed in the laboratory ever since. It can therefore be assumed that there is hardly any genetic variation between wasps within these three strains.

Creating size variation

Differentially sized *Trichogramma evanescens* wasps (Figure 1) were reared using different butterfly host species. In order to get small wasps, females were given the opportunity to parasitize small eggs of the Mediterranean flour moth *Ephestia kuehniella*. Females were left together with a small amount of butterfly eggs, to have them deposit relatively many wasp eggs per host egg (most likely 2 *T. evanescens* wasps developing inside one *E. kuehniella* egg). This superparasitism should result in very small wasps. Superparasitism has a risk of shifting the sex ratio of the emerging wasps to a more male biased population (Wajnberg and Hassan, 1994). As females were used for the analysis, this was corrected for by ensuring that a relatively large amount of eggs were parasitized. Larger wasps were reared by allowing the wasps to parasitize much larger eggs of the small cabbage white butterfly *Pieris rapae*, the large cabbage white butterfly *Pieris brassicae* and the cabbage moth *Mamestra brassicae*. Eggs of *P. brassicae* and *P. rapae* were largest, but because these eggs were deposited respectively in small clutches and as single eggs on leaves of Brussels sprouts plants, they were difficult to use in high quantities in rearing. Therefore these eggs were used in combination with eggs of *M. brassicae*, which were slightly smaller, but easier to handle due to the large size of their egg clusters and the fact that these eggs were deposited on sheets of paper. Wasps emerging from eggs of these three host species were rather large when a relatively small number of females had been allowed to parasitize the eggs (resulting in few wasps developing per egg), whereas superparasitism also resulted in very small wasps on these host species (many wasps developing per egg). This breeding procedure was done every other day to have enough wasps present of various body sizes when these were needed. When it became clear that wasps emerging from *M. brassicae* eggs were not notably smaller than wasps that developed in eggs of *P. brassicae* and *P. rapae*, rearing continued using *M. brassicae* and *E. kuehniella* solely. In order to get

the largest wasps possible using *P. rapae*, *P. brassicae* and *M. brassicae* eggs as host species, the ovipositing behaviour was observed as described previously (Figuerola, 2010; Huigens et al., 2004; Huigens et al., 2000; Suzuki et al., 1984) to make sure that only one fertilised egg was laid in each butterfly egg. The observations of the ovipositing behaviour were performed only a few times, because of the large amount of time it took and only a small number of very large wasps was needed.

Preparing wasps for confocal laser scanning microscopy using GPA

Wasps that apparently differed in body size were immersed in GPA fixative (5% glutaraldehyde, 75% picric acid (saturated aqueous solution), 2% acetic acid) (Boer et al., 1979) in a dissection tray, and cut in the abdomen in order to let the fixative infiltrate. After six hours of infiltration the wasps were rinsed three times using 70% ethanol, until the yellow colour of the GPA had been cleared from the dissection tray. They were kept in ethanol overnight. The next day wasps were dehydrated in graded ethanol concentrations (5 minutes of 90%, 96% and twice in 100% ethanol) and decapitated. Heads and bodies were stored separately in 100% ethanol in a refrigerator until further use.

Preparing wasps for confocal laser scanning microscopy using FMP

At first I was planning to primarily relate hind tibia length (a commonly used parameter for size in parasitic wasps (Kazmer and Luck, 1995)) as a parameter for body size to brain volume using GPA as fixative. Unfortunately, such a relationship proved to be difficult to compare to brain/body size relationships for other animals described in literature because most other studies used brain and body weights instead of linking a three dimensional volume to a one dimensional length. Since *Trichogramma evanescens* wasps are too light to weigh on any in the laboratory available scale, the decision was made to perform additional experiments, this time keeping the abdomens of the wasps intact in order to measure body volume as well.

The wasps that were used to measure head, brain and body volumes were treated with the fixative FMP (4% formaldehyde in 50% MeOH 0.1M phosphate buffer), that assumed to have an enhanced penetration capacity in the absence of an abdominal cut compared to GPA. Because of the high crystallisation rate of the fixative, a fresh stock of FMP was made right before every fixation. Both head and body shape had to remain intact, therefore this time no cuts were made in the wasps' abdomens. In order to be sure that the wasps were fixated properly despite this lack of large openings in the tissue, they were left in the fixative overnight. A longer fixation time was not possible, because of the formation of crystals. Rinsing and dehydration steps were the same as with the GPA fixation. After the last step,

wasps were kept in 100% ethanol, in which their heads and hind legs were removed. Heads and bodies were stored together in 100% ethanol in a refrigerator. Hind legs were stored separately until measurement of tibia length.

Microtome sectioning of heads for light microscopy

Confocal microscopy does not allow a view of the structures underneath the light reflecting eye pigments (Figure 2b). In order to make sure that the brain occupies the whole area between the eye pigments and the visible areas of the brain, some heads were prepared for conventional light microscopy. For this purpose, heads fixed in GPA were taken out of the cool storage and immersed in Technovit 7100 infiltration solution (made by solving 1 g hardener I in 100 mL Technovit 7100 base liquid (Heraeus Kulzer GmbH & Co.)) for 24 hours. The next day, a polymerisation solution (consisting of 15 mL infiltration solution and 1 mL of hardener II) was made and poured into the holes of a Histoform S embedding form. In each hole a *T. evanescens* head was placed with the front of the head facing upwards. This was always done within 5 minutes, since the two hardeners started to polymerise immediately. When the polymerisation in the form was complete, a Kulzer Histobloc was placed on each form and mounted using Technovit 3040 two component resin (using a 1:1 volume mixing ratio of powder and liquid). After the resin had completed polymerisation, the Histobloc was removed from the form. Using a rotation microtome equipped with Feather disposable knife blades 5 µm thick sections

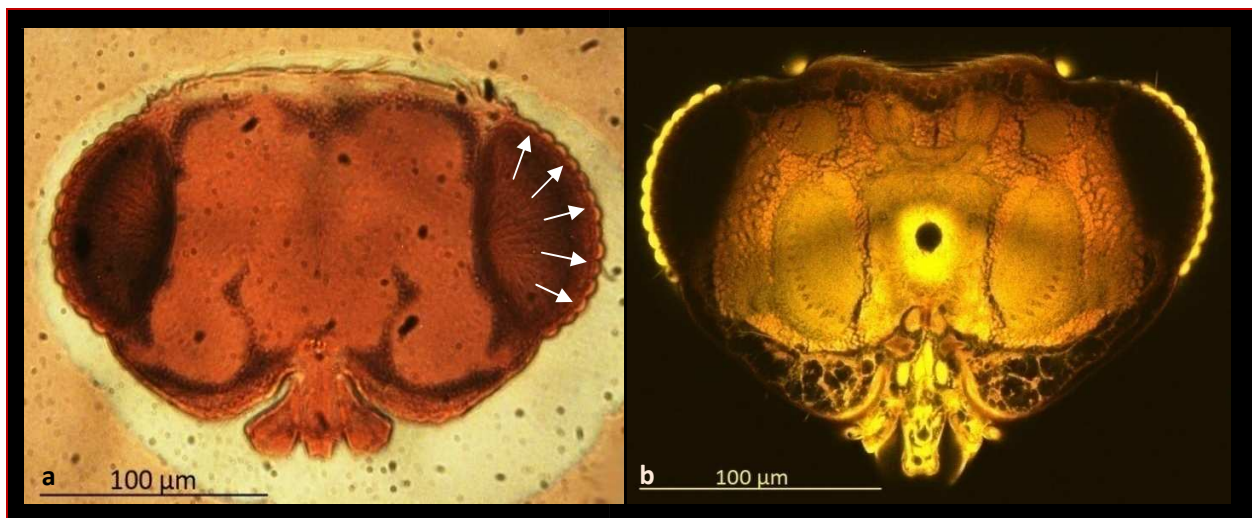


Fig. 2. Cross sections through the head of a *Trichogramma evanescens* wasp. **(a)** Microtome sliced head dyed with eosin-haematoxylin, as seen using light microscopy. **(b)** Confocal laser scanning microscope (CLSM) picture of an unstained, intact head. Light microscope picture reveals that the entire area between the eye pigments and brain centre, some of which is covered by a shadow on the CLSM pictures, consists of brain tissue. Arrows show where the optic lobe's lamina is attached to the eye pigments.

were made of the *T. evanescens* heads. Each slice was captured from the microtome knife using two pairs of tweezers and put in a drop of water on a glass microscope slide. The slide was thereafter placed on a warm plate to let the sections stretch and dry. The sections were stained in an eosin-haematoxylin (Sigma-Aldrich Co.) solution for 8 minutes, dried and mounted in DPX under a cover slide. Pictures were taken using a camera on a light microscope (Figure 2a).

Measurement of hind tibia length

Hind legs of the stored wasp bodies were removed using a stereo microscope and sharpened tweezers. Hind tibia length was measured under a light microscope (400x magnification) with an ocular lens with measuring line. Wasps were selected for further analyses based on their tibia lengths, thereby making sure that a large variety of tibia lengths was present in the selection, and that small and large wasps were equally well represented. For both the GPA and the FMP method, 25 wasps per strain were selected, leading to 75 wasps per fixative. When some wasps had to be left out because of cuts in the tissue or other artefacts, few extra wasps were selected to make sure that there were still at least 75 wasps in the sample. Eventually, the GPA sample size consisted of 77 intact wasps and the FMP sample of 87.

Volume measurements

The heads and bodies were placed in pure xylene for approximately 20 minutes to make the tissue transparent. During this time the antennae and remaining legs and wings were removed with sharp tweezers, to make sure that the heads and bodies would not tilt during mounting. The transparent heads and bodies were mounted in a drop of DPX on a microscope glass. Heads were placed with the neck upwards, to minimise the size of the shadow of the eye pigments on the picture. Consequently, a slight shadow of the tracheoles in the back of the head was cast, but this shadow did not block the image of underlying tissues and did therefore not interfere with the volume measurements. Bodies were placed on their side, which resulted in a better visible and understandable image than a ventral or dorsal placement. To prevent any deformations caused by the pressure of the microscope glass, the heads and bodies were mounted with extreme care. These objects were scanned with a Zeiss LSM 510 confocal laser scanning microscope (CLSM) using a 488 nm wavelength argon laser and a Plan-Neofluar 40x oil-immersion objective for the heads and a Plan-Apochromat 10x lens for the bodies. The digital image stacks were saved and analysed using Amira 5.3 software (Visage Imaging GmbH), which enables the production of three dimensional reconstructions (Figure 3) of the wasps' brains, bodies and heads. These

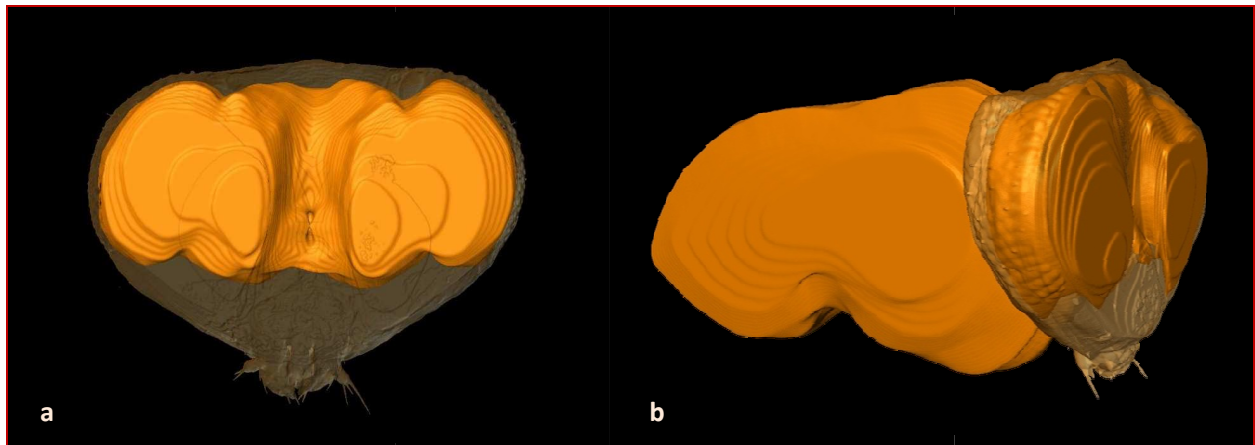


Fig. 3. Three dimensional reconstructions of a *Trichogramma evanescens* wasp. **(a)** Frontal view of head capsule (shown transparent) with brain inside. **(b)** Combined reconstruction of head (with brain inside) and body.

reconstructions were subsequently used to calculate brain, head and body volumes with the material statistics option in the software. To create the three dimensional reconstructions, outlines of brains, heads and bodies had to be drawn on every picture of the image stacks using the segmentation editor of the Amira 5.3 software. The shadows underneath the eye pigments were also counted as part of the brain, because the analysed slices of the *Trichogramma evanescens* heads under the light microscope showed that the only structure present underneath the eye pigments is the optic lobe (Figure 2a). To obtain the volume of the entire body, the volume of the head was combined with the volume of the thorax and abdomen. Using this method, body volume was estimated in a rather accurate way. When even larger individuals were analysed, the laser might not be able to penetrate the tissue entirely and it might be necessary to scan the objects from both sides and merge the resulting pictures in Amira 5.3 software.

Measurement of head width and body length

Head width and body length were measured with the three dimensional line measuring tool in the Amira 5.3 software. Head width was defined by the largest straight line taken from one side of the head (using the eye pigment as a border) to the other side (Figure 4a). Body length was defined by the distance following the body axis from the tip of abdomen to the end of the thorax (Figure 4b). Both were measured in the optical section where head width and body length were maximal, using the three dimensional reconstruction to determine the right orientation.

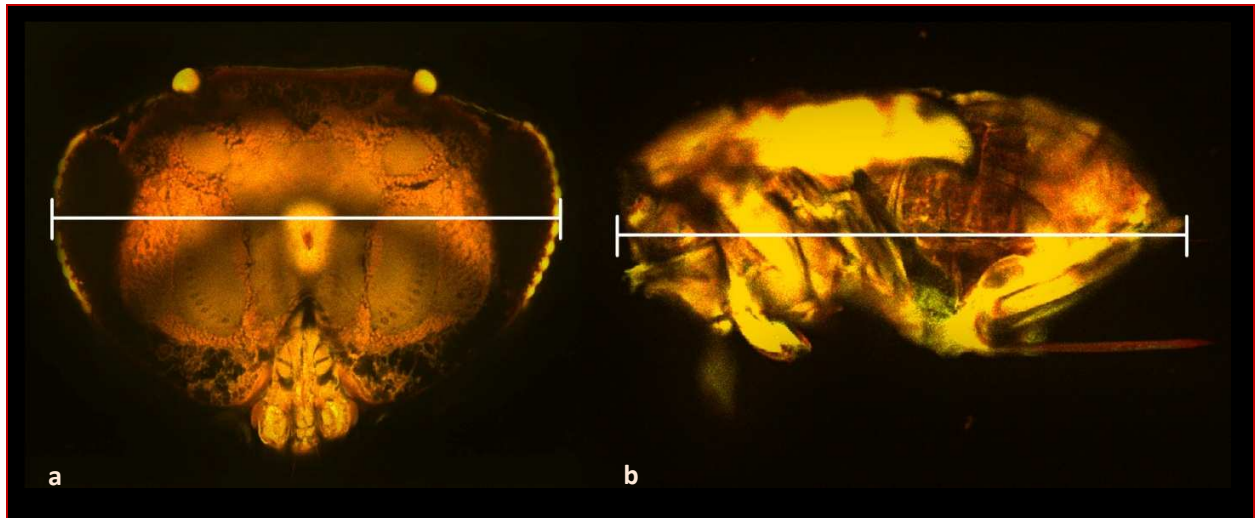


Fig. 4. Definitions of **(a)** head width and **(b)** body length. Pictures were taken with a confocal laser-scanning microscope. Both lengths were measured using the Amira 5.3 software.

Statistical analysis

The natural logarithms of the body size parameters were calculated to obtain linear relationships of the power functions describing the relationships between body size and brain volume. This transformation allowed the use of least-squares regression analyses on the dataset to find the precise relationships with brain volume and body size parameters. Several methods of regression are applied in allometry studies to account for the variation in both body size and brain size (Warton et al., 2006), but for intraspecific relationships least-squares regression is usually applied, since these data are assumed to follow a bivariate normal distribution (Wehner et al., 2007).

To test if the wasps' genotype or host species influenced the relationship between brain volume and body volume, an ANCOVA test was used with the natural logarithm of body volume as covariate and the specific wasp strains or host species as a fixed factor. In the test for influences of host species, the entire sample of wasps reared on *Ephestia kuehniella* was compared to the entire sample of *Mamestra brassicae* reared wasps. A second analysis compared wasps with body sizes within the range that was present in both *E.*

T-test for comparing two regression slopes:

$$t = \frac{b_1 - b_2}{s_{b_1 - b_2}} \quad \text{with} \quad s_{b_1 - b_2} = \sqrt{s_{b_1}^2 + s_{b_2}^2}$$

T-test for comparing a regression slope against 1:

$$t = \frac{b_1 - 1}{s_{b_1}}$$

Box 1. Manual Student's T-tests for comparisons of regression slopes. b_1 is the slope of regression line 1, which has a standard error of s_{b_1} . b_2 is the slope of regression line 2, which has a standard error of s_{b_2} . $s_{b_1 - b_2}$ is the standard error of the difference between the two slopes. P-values can be calculated using the t-value and the degrees of freedom.

kuehniella and *M. brassicae* reared wasps: $2.249 * 10^6 - 4.745 * 10^6 \mu\text{m}^3$, thereby excluding the smallest wasp that developed inside a *E. kuehniella* egg and the largest ones emerging from *M. brassicae* eggs. All of these statistical tests were performed in SPSS 16.0.

To test for allometry in the regression slopes, the slopes were compared to the value 1 using Student's t-test (Box 1). The same test was also used to compare two regression slopes, produced after using the two different fixation methods, to each other.

Results

Brain allometry

The relationship between brain volume and body volume in *Trichogramma evanescens* was found to be significant ($n=87$; $F_{1,85}=764.5$; $p<0.001$) and had a regression of $y = 0.911x - 0.679$ ($R^2 = 0.8999$), thus showing a weak negative allometric relationship (Figure 5b, untransformed values shown in Appendix Figure 12). The slope of the regression line was much larger than expected and rather close to 1, which might indicate an isometric relationship between brain size and body size. Therefore, the slope of the regression line was tested against 1 and the relationship appeared to deviate significantly from isometry ($t(85) = -2.697$; $p<0.01$). This weak negative allometry is also shown in figure 6, as there is a slight decrease in relative brain volume (the proportion of body volume that consists of brain volume) with increasing body volume, showing a significant linear regression of $y = -0.0116x + 0.310$ ($n=87$; $F_{1,85}=7.265$; $p=0.008$; $R^2 = 0.0787$). This figure also illustrates the large variability in relative brain volume for wasps, which was found to range between 9.19 – 17.50 % of body volume. On average, brain volume constitutes 13.09% of total body volume in *T. evanescens* (95% confidence limits: 12.70 – 13.48), which indicates a large relative brain size.

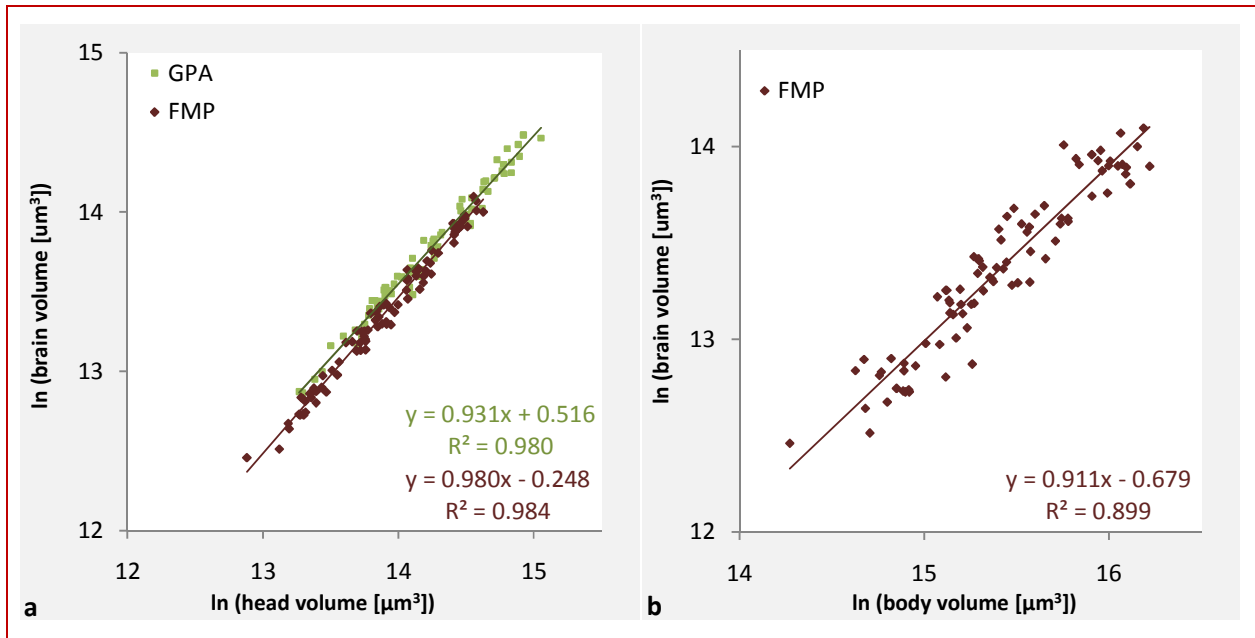


Fig. 5. Scatterplots showing the relationship between (a) brain volume and head volume and (b) brain volume and body volume. Green squares show data obtained using GPA as fixative ($n=77$). Red diamonds show wasps treated with FMP ($n=87$).

Another finding in this study is the large variation in absolute body and brain size in *T. evanescens* wasps, depending on the amount of nutrition that was available during the wasps' development. When wasps from all analysed strains were combined, brain volumes were found to vary intraspecifically by an order of magnitude of more than 5 (Table 1). Phenotypic variations in brain volume within the three iso-female strains were slightly lower, but still substantial. In the sample of wasps treated with FMP, a factor of almost 4 was found in the two wasp strains GD011 and GD034, yet brain volume varied even by an order of magnitude of 5 in strain GD025. The largest *T. evanescens* wasps had body length parameters of twice the size of the smallest wasps and consequently a body volume more than 7 times as large as that of the smallest wasps.

The relationship between brain volume and head volume was expected to be isometric, because the volume of the head most likely mainly depends on the size of the brain that is inside. The regression line for the relationship between brain and head volume obtained from GPA treated wasps (Figure 5a, green line) was highly significant ($n=77$; $F_{1,75}=3592.7$; $p<0.001$) and can be described by $y = 0.931x + 0.516$ ($R^2 = 0.980$). A more or less similar relationship was found for the FMP treated wasps (Figure 5a, red line), also highly significant ($n=87$; $F_{1,85}=5161.5$; $p<0.001$) and described by $y = 0.980x - 0.248$ ($R^2 = 0.984$). There was a small but significant difference between the two slopes ($t(162) = -2.305$; $p = 0.022$). The GPA regression deviates significantly from isometry ($t(75) = -4.312$; $p<0.001$), whereas the slope obtained using FMP as fixative does not differ significantly from 1 ($t(85) = -1.429$; $p=0.157$) and is thus isometric.

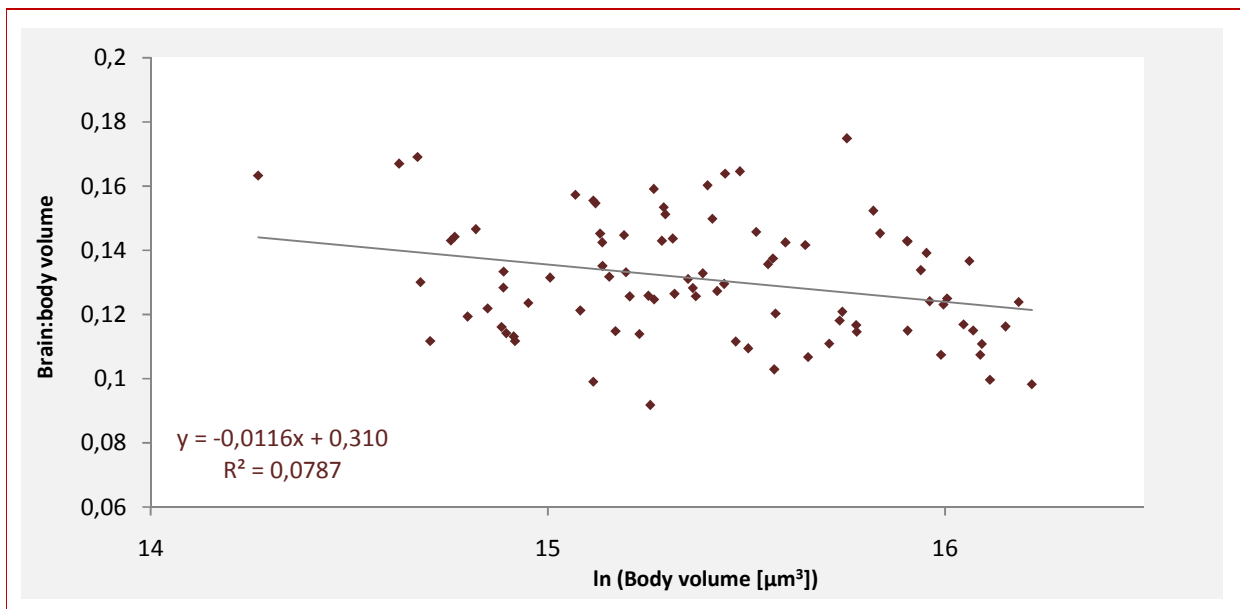
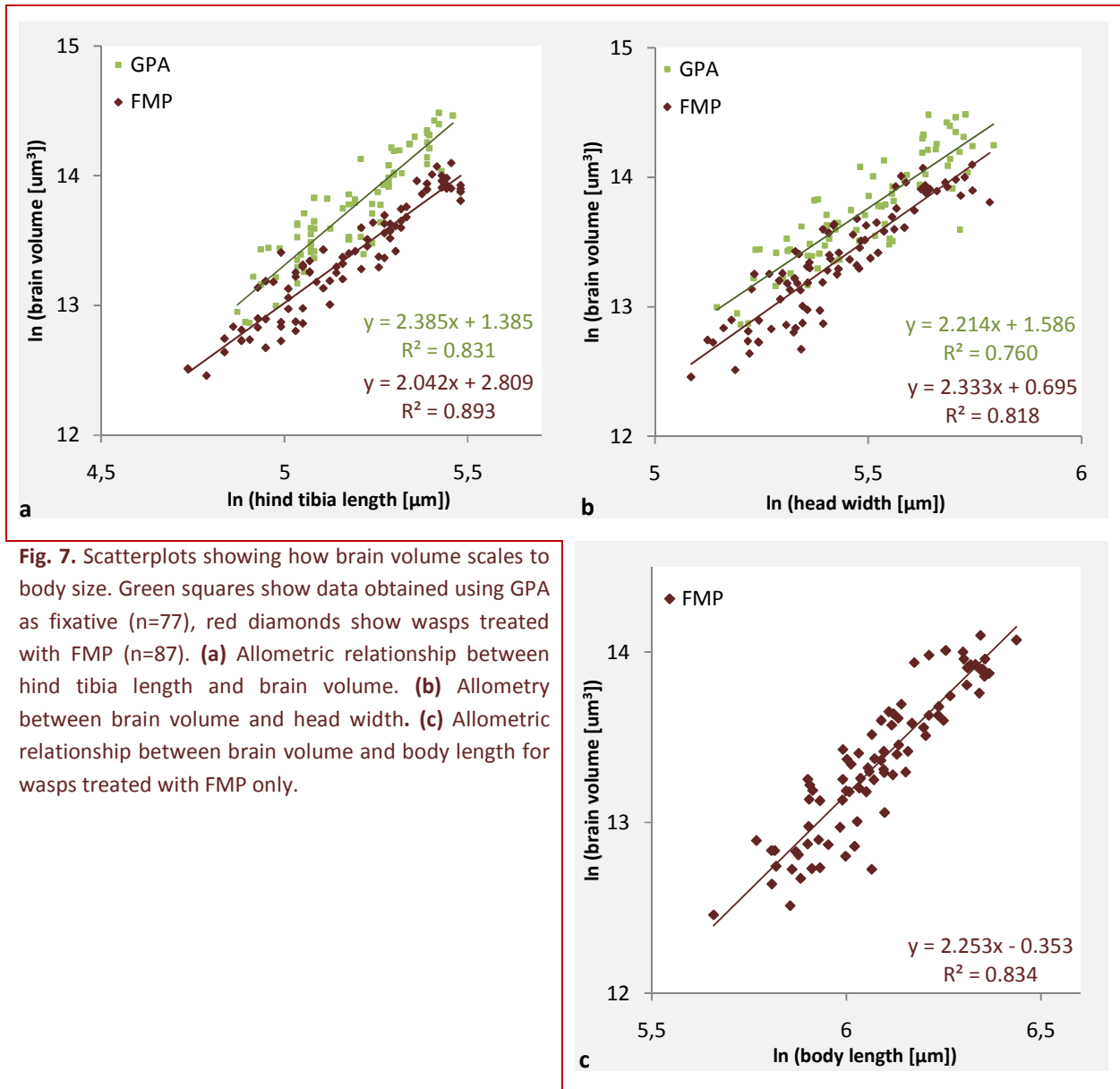


Fig. 6. Scatterplot of the ratio between brain volume and body volume against body volume for *Trichogramma evanescens* ($n=87$).

Table 1. Size variation in *Trichogramma evanescens* treated with FMP as fixative. Size ranges, mean values and the factor of multiplication (largest value / smallest value) are shown for all wasp strains (n=87).

	Range	Mean	Factor
Body length [μm]			
<i>GD011</i>	335 – 582	447	1.74
<i>GD025</i>	287 – 625	448	2.18
<i>GD034</i>	359 – 576	447	1.60
<i>Total</i>	287 – 625	448	2.18
Head width [μm]			
<i>GD011</i>	168 – 325	240	1.93
<i>GD025</i>	162 – 304	232	1.88
<i>GD034</i>	178 – 283	223	1.59
<i>Total</i>	162 – 325	231	2.01
Hind tibia length [μm]			
<i>GD011</i>	126 – 240	180	1.90
<i>GD025</i>	114 – 234	171	2.05
<i>GD034</i>	132 – 231	178	1.75
<i>Total</i>	114 – 240	179	2.11
Brain volume [μm^3]			
<i>GD011</i>	336,389 – 1,326,117	716,775	3.94
<i>GD025</i>	257,547 – 1,291,430	684,128	5.01
<i>GD034</i>	319,101 – 1,214,324	682,083	3.81
<i>Total</i>	257,547 – 1,326,117	694,328	5.15
Head volume [μm^3]			
<i>GD011</i>	583,003 – 2,250,738	1,244,527	3.86
<i>GD025</i>	393,102 – 2,133,207	1,159,492	5.43
<i>GD034</i>	533,879 – 2,139,333	1,152,937	4.01
<i>Total</i>	393,102 – 2,250,738	1,185,651	5.73
Thorax + abdomen volume [μm^3]			
<i>GD011</i>	2,202,944 – 9,177,094	4,551,742	4.17
<i>GD025</i>	1,183,666 – 7,892,262	4,292,933	6.67
<i>GD034</i>	2,045,165 – 7,495,408	3,806,781	3.66
<i>Total</i>	1,183,666 – 9,177,094	4,217,152	7.75
Body volume [μm^3]			
<i>GD011</i>	2,809,226 – 11,055,407	5,796,269	3.94
<i>GD025</i>	1,576,768 – 9,746,191	5,452,424	6.18
<i>GD034</i>	2,671,736 – 9,307,497	4,959,718	3.48
<i>Total</i>	1,576,768 – 11,055,407	5,402,803	7.01



The relationship between hind tibia length and brain volume is significantly described by $y = 2.385x + 1.385$ ($R^2 = 0.831$) for GPA (Figure 7a, green line) (n=77; $F_{1,75}=369.8$; $p<0.001$). This relationship was also significant for wasps treated with FMP (n=87; $F_{1,85}=707.8$; $p<0.001$) and can be described by $y = 2.042x + 2.809$ ($R^2 = 0.893$) (Figure 7a, red line). There was a significant difference between the two slopes ($t(162) = -7.742$; $p<0.001$), indicating a slightly different relationship between tibia length and brain volume due to the fixative used.

The relationship between brain volume and head width (Figure 7b) has a regression model of $y = 2.214x + 1.586$ ($R^2 = 0.760$) for the GPA treatment (green line) and a model of $y = 2.333x + 0.695$ ($R^2 = 0.818$) for the FMP treatment (red line). Both models were significant (for GPA: $n=77$; $F_{1,75}=237.1$; $p<0.001$ and for FMP: $n=87$; $F_{1,85}=380.8$; $p<0.001$), but there was no significant difference between the two slopes ($t(162)=-0.635$; $p=0.526$). The R^2 is highest in the correlations between brain volume and hind tibia length (GPA: $R^2 = 0.831$ and FMP: $R^2 = 0.893$), suggesting that brain volume can be estimated more accurately when measuring hind tibia length than when using head width.

The relationship between brain volume and body length (Figure 7c) could be made for wasps treated with FMP only. The relationship was significant ($n=87$; $F_{1,85}=426.9$; $p<0.001$) and can be described by $y = 2.253x - 0.353$ (blue line) ($R^2 = 0.834$).

Genotypic effects

The allometric relationships between brain volume and body volume (Figure 8) were significant for all tested strains of *Trichogramma evenescens* (for GD011: $n=29$, $F_{1,27}=354.5$, $p<0.001$; for GD025: $n=29$, $F_{1,27}=360.2$, $p<0.001$; and for GD034: $n=29$, $F_{1,27}=227.6$, $p<0.001$). Whereas both GD011 and GD025 show a weak negative allometric relationship (GD011: $y = 0.925x - 0.911$, $R^2 = 0.929$ and GD025: $y = 0.820x + 0.729$, $R^2 = 0.931$), the regression of GD034 showed positive allometry ($y = 1.118x - 3.811$, $R^2 = 0.894$, 95% confidence intervals for b : $0.966 - 1.270$), suggesting that in this strain large wasps have relatively slightly larger brains than small wasps. To test for the significance of these deviations from isometry, all slopes were tested against 1. This showed that the deviations were not significant for both GD011 and GD034 ($t(27)=-1.551$, $p=0.125$ and $t(27)=1.595$, $p=0.115$ respectively), which had slopes closest to 1. The regression of GD025, however, did deviate significantly from isometry ($t(27)=-4.186$, $p<0.001$).

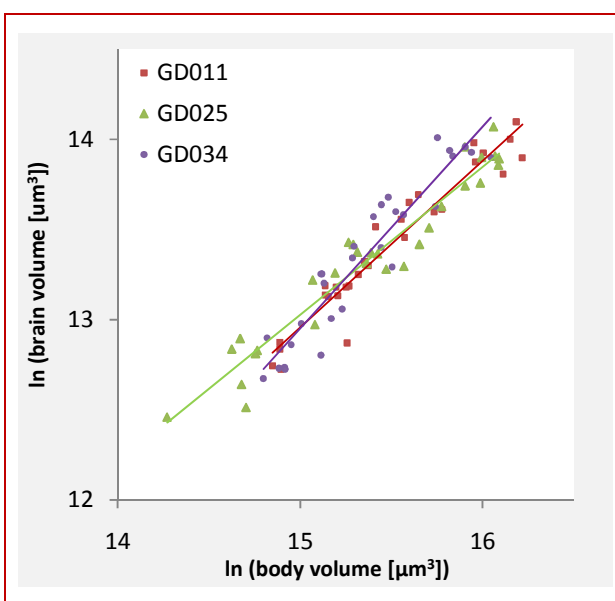


Fig. 8. Relationship between brain volume and body volume for the iso-female wasp strains GD011 (red squares), GD025 (green triangles) and GD034 (purple circles). All strains had a sample size of 29 wasps.

The ANCOVA analysis revealed that even though the covariate, the natural logarithm of the wasps' body volume, was significantly related to the natural logarithm of brain volume ($F(1,83)=773.468$, $p<0.001$), there was no significant effect of the wasps' genotype (the fixed factor) on brain volume ($F(2,83)=1.550$, $p=0.218$). This result shows that there is no significant difference between relationships of the three strains.

Host effects

Wasps bred on *Ephestia kuehniella* and *Mamestra brassicae* were compared to each other to check for differences in their relationship between brain volume and body volume. This was done by comparing all wasps bred on *E. kuehniella* to all wasps bred on *M. brassicae* (Figure 9a) and by comparing all wasps that had body volumes represented in both the *E. kuehniella* and the *M. brassicae* reared sample of wasps, i.e. the smallest wasps that developed in *M. brassicae* eggs and the largest of *E. kuehniella* reared wasps (Figure 9b).

All relationships were significant (for *Ephestia*_{all}: $y = 0.995x - 1.961$, $n=29$, $F_{1,27}=105.8$, $p<0.001$, $R^2=0.797$;

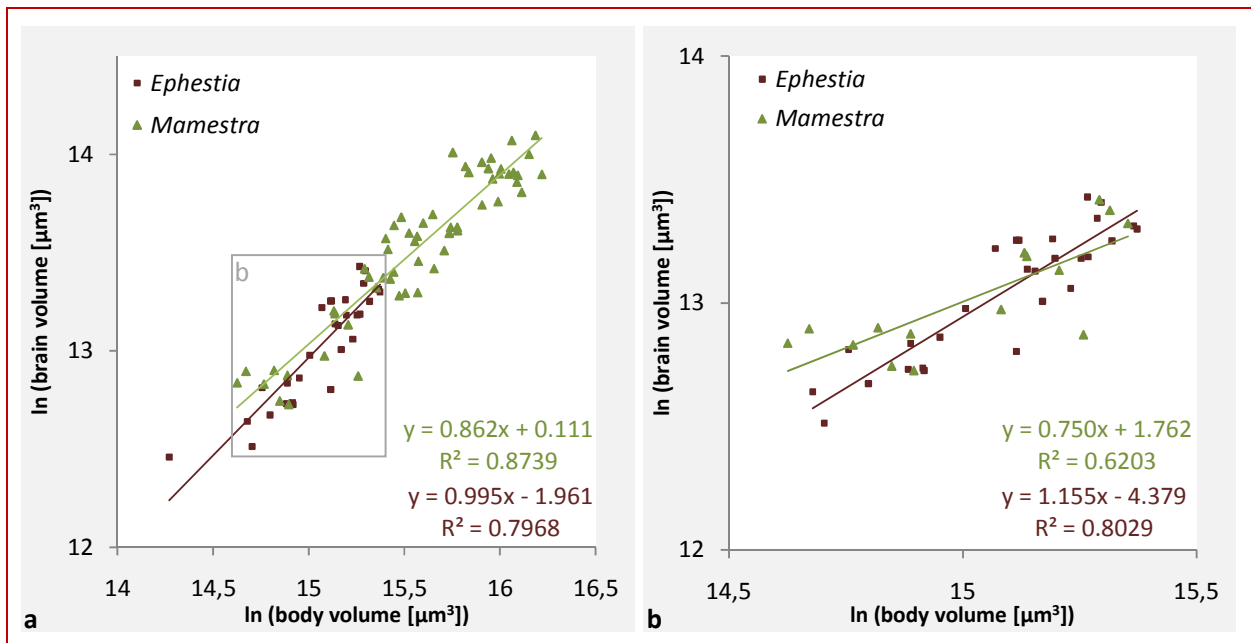


Fig. 9. Scatterplots showing the relationship between brain volume and the volume of the combined head and body volumes for the two different hosts, showing *Ephestia kuehniella* bred wasps as red squares and *Mamestra brassicae* bred wasps as green triangles, for **(a)** the entire sample ($n_{\text{Ephestia}}=29$ and $n_{\text{Mamestra}}=58$) and **(b)** for wasps bred on *E. kuehniella* ($n=28$) and wasps bred on *M. brassicae* ($n=15$), which had body volumes present in both samples ($2.249 \times 10^6 - 4.745 \times 10^6 \mu\text{m}^3$), as shown by the square in **(a)**. FMP was used as fixative.

for *Ephestia*_{matched}: $y = 1.155 x - 4.379$, $n=28$, $F_{1,26}=105.895$, $p<0.001$, $R^2=0.803$; for *Mamestra*_{all}: $y = 0.862x + 0.111$, $n=58$, $F_{1,55}=388.2$, $p<0.001$, $R^2=0.874$ and for *Mamestra*_{matched}: $y = 0.750 x + 1.762$; $n=15$; $F_{1,13}=21.238$; $p<0.001$; $R^2=0.620$).

Similar as in the test for genotypic differences, an ANCOVA analysis was performed to test if there was a significant effect of the wasps' host species on brain volume after controlling for the effects of body volume. This test showed that host species does not significantly influence the relationship between brain volume and body volume when testing wasps with body volumes within the body size range that was present in both host species samples ($F_{2,40}=0.909$; $p=0.346$), nor when testing all *E. kuehniella* bred wasps against the entire *M. brassicae* sample ($F_{2,84}=1.818$, $p=0.181$).

Discussion

Brain allometry

I expected small *Trichogramma evanescens* wasps to have brains that are relatively much larger than those of large wasps, because *T. evanescens* wasps have minute brains, which could be on the edge of what is physiologically possible. Their brain size reduction might be limited by a minimum size and number of neurons vital for normal brain functions and wasp performance. The relationship I expected to find would show as a strong negative allometric relationship between body size and brain size in *Trichogramma evanescens*, in which the scaling coefficient b approaches 0 and body size has hardly any influence on the size of a wasp's brain.

In contrast to this prediction, *Trichogramma evanescens* wasps display the exact opposite: a very weak negative allometric relationship between brain volume and

body volume that approaches isometry. Whereas other studies of intraspecific brain allometry in insects find slopes ranging between 0.2 – 0.4 (Riveros and Gronenberg, 2010; Seid et al., 2011; Wehner et al., 2007), the scaling coefficient is approaching 1 in *T. evanescens* (Figure 6b).

This discrepancy is illustrated in figure 10, where intraspecific relationships are shown for workers of three *Cataglyphis* ant species (Wehner et al., 2007). This figure clearly shows that the intraspecific relationships found for ants are less steep than the one found for *T. evanescens*, which has a scaling coefficient approaching 1 whereas the b of ants seems to approach 0. The difference between the ant slopes and the one for *T. evanescens* illustrates their different brain scaling strategies. In ants, the largest individuals of a species clearly have relatively much smaller brains than their small conspecifics, whereas

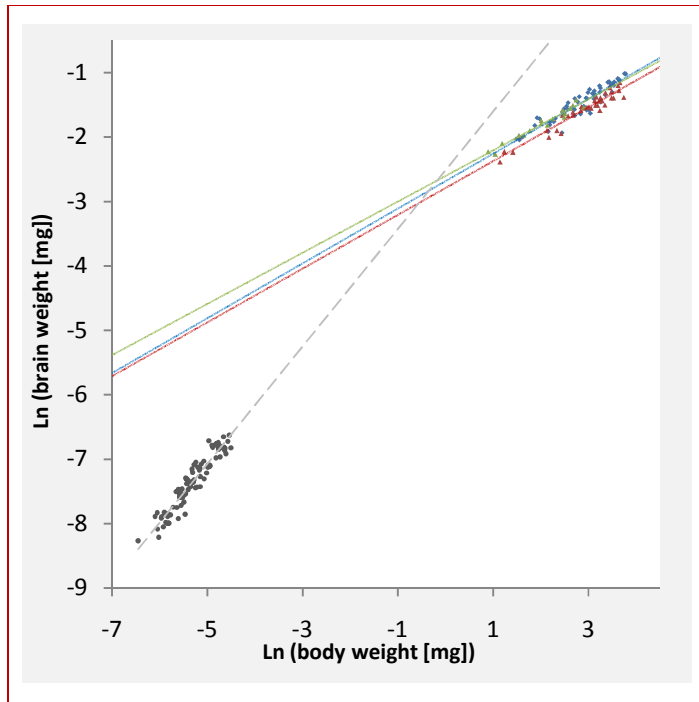


Fig. 10. Scatterplot showing a comparison between three *Cataglyphis* ant species and *Trichogramma evanescens*. Scaling coefficients for the ant species: *C. bicolor* $b=0.42$ (blue line), *C. mauritanicus* $b=0.40$ (red line) and *C. viaticus* $b=0.39$ (green line). The coefficient for *T. evanescens* (dashed grey line) is more than twice as large, $b=0.91$. Volume data of *T. evanescens* was converted to mass assuming a density of 1 g/ml. Intraspecific data for the ant species from Wehner et al., 2007.

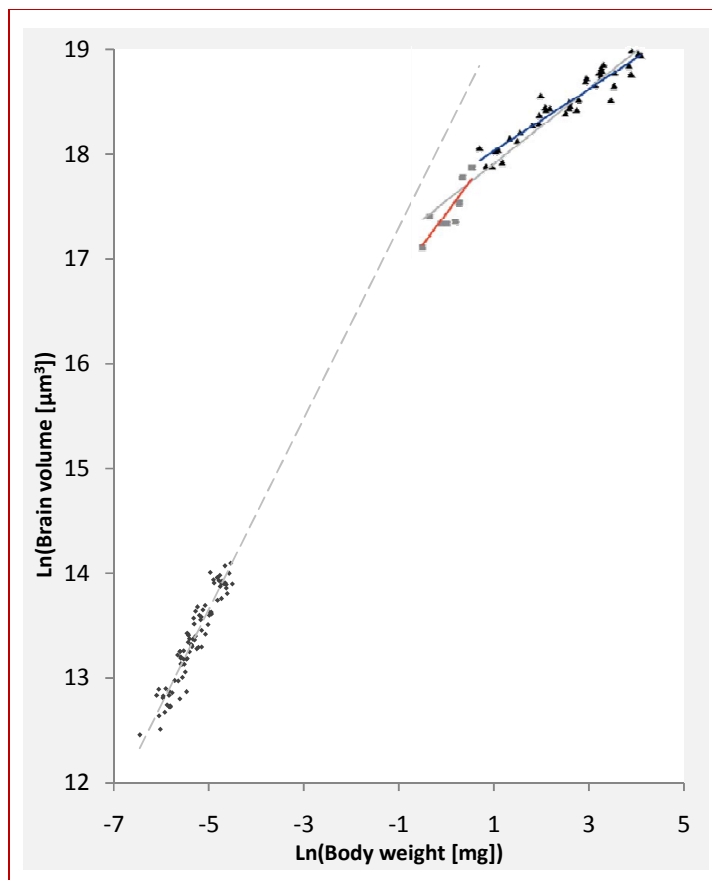


Fig. 11. Scatterplot showing a comparison between *Trichogramma evanescens* (grey line) and *Atta colombica* ants (red and blue lines). The scaling coefficients of the large ants is $b=0.29$, for the smallest ants of the same species the coefficient is $b=0.60$. The scaling coefficient for *T. evanescens* is $b=0.91$. Body volume data of *T. evanescens* were converted to mass assuming a body density of 1 g/ml. Ant data from Seid et al., 2011.

and body size is not entirely fixed, but allows some flexibility. There is hardly any genotypic variation within the iso-female strains, but this variation might be sufficient to explain the (weak) negative allometry, i.e. sufficient to explain the slight deviation from isometry. Larger wasps could show slightly smaller brains than expected from isometry to compensate for the high energetic costs of brain tissue and the relatively small benefits of having a larger brain.

Second, it could be that the wasps' brains are as large as possible relatively to their body size. Having a large brain requires a substantial investment in energetically costly brain tissue. To acquire this large amount of energy, wasps may need an increased energy intake or decreased amount of energy available for reproduction and maintenance compared to animals with relatively smaller brains (Isler and van

in *T. evanescens* the large wasps have only slightly smaller relative brain sizes compared to small wasps. Also, in ants body size seems barely to influence brain size, while body size determines brain size almost completely in *T. evanescens*.

There are several possible explanations for this relationship's resemblance to isometry in *T. evanescens*, all based on a rather fixed ratio between brain size and body size. First, the brain:body ratio might be fixed in the wasps' genotypes. In that case, an isometric relationship would be observed between brain size and body size of wasps of the same iso-female strain. There could be differences in the intercept between the strains, accounting for the absolute differences in the brain:body ratio between the genotypes, but within a strain hardly any variation in the ratio should be present. A weak negative allometric relationship would be observed if the ratio between brain size

Schaik, 2009). A relatively even larger brain may thus result in costs that will become too high to overcome at a certain point. This explanation would also expect brain isometry if it would still be advantageous for the largest wasps to have a brain that is as large as possible. If, at some point, a further absolute increase in brain size would result in energetic costs that are too large to overcome the relatively few further improvements of the wasps' brain functions, this would result in a weak negative allometric relationship. Large wasps, with a slightly smaller brain:body ratio than small wasps, would then be able to increase the relative amount of energy available for reproduction to improve their fitness, as it has been observed that large *T. evanescens* wasps produce more offspring than small wasps (Doyon and Boivin, 2005). This could be caused by a decreased amount of energy necessary to maintain their brains compared to their smaller conspecifics that are relatively larger brained.

In my study, relative brain volume was highly variable, ranging between 9 and 17 % of body volume (Figure 5). This seems to contradict the two explanations stated before, which were both based on a rather fixed ratio between brain size and body size. However, the relationship as illustrated in the figure does show that the ratio is fixed between 9 and 17 %, as it does not get larger or smaller than those values. In interspecific relationships in ants, relative brain sizes can range between 0.5 and 15% (Seid et al., 2011), thus showing clear negative allometry as these ratios become smaller with increasing body size. The variability in the brain:body ratio observed in my samples indicates that there is some flexibility in the relative brain size of *T. evanescens* wasps, explaining their weak negative brain allometry.

Although the observed scaling coefficient within *T. evanescens* is exceptionally large, it is not the first time a large coefficient has been shown in small insects. Seid et al. (2011) found that small ants show a larger allometric slope than larger ones, both inter- and intraspecifically. Figure 11 shows a comparison between the intraspecific data as presented in their paper and the data found for *T. evanescens* in this thesis. Whereas the overall scaling coefficient of brain allometry in *Atta colombica* is 0.35, Seid et al. found that it reaches 0.60 in the smallest individuals of the species. This means that the smallest ants had even smaller brains than was expected from the overall regression line of the species. The authors explained this as a compensation for the otherwise too costly nervous system, or by a possible increase of the small ants' peripheral sensory systems to enable them to relocate certain brain functions to other parts of the nervous system.

Here, we are looking at even smaller brain sizes, among the smallest in the Animal Kingdom, which still enable their owners to perform rather complex cognitive functions. The possible explanations by Seid et al. for the undersized brain volumes of the small *Atta colombica* ants should also be investigated for *Trichogramma evanescens*, as it might be possible that these wasps are able to show minute brain sizes

by means of a relatively large investment in their peripheral sensory system, thereby moving some of their brain functions from the brain tissue to the sensory periphery or to nervous tissue in the abdomen. Another possibility is that a small brain size is reached because the size of *Trichogramma evanescens*' neurons are smaller than in other insects, in which neuron soma diameter of Kenyon cells in the mushroom bodies is limited to 3-8 μm (Laurent and Naraghi, 1994). First octopamine labelling and CLSM analyses suggests the diameter of these cells in *T. evanescens* to be only 1 μm (F. Pashalidou, M.E. Huigens & H.M. Smid, unpublished data). This could enable a linear scaling of brain size in which neuron number is the same in the small and large brains and might allow the wasps to reach their minute body and brain sizes without having to compromise brain performance.

If small animals indeed show larger scaling coefficients than large ones, as was shown by Seid et al. (2011), it makes sense to observe an even larger coefficient in *T. evanescens*. This trend, as illustrated in figure 11, can be further explored by rearing even larger *T. evanescens* wasps by means of larger host eggs than those used in this study, for example those of the tobacco hornworm *Manduca sexta*. The scaling coefficient of the largest possible *T. evanescens* wasps could already be smaller than the one observed in this study, which can be shown using piecewise regression analyses (as performed for my sample in the appendix, section 'diphasic allometry in small and large wasps').

Body size variation

Another important observation in this study is the large phenotypic variation in absolute brain volume, solely based on host egg size and the number of developing wasps inside it. Brain volume was found to vary by a factor of almost 4 in two of the three iso-female strains, and even exceeding 5 in the other (Table 1). Since the wasps within a strain are assumed to share the same genotype, these large variations in both body and brain volume are phenotypic. No literature was found on phenotypic variations of brain size in genetically identical insects were studied, but some enormous intraspecific brain volume variations have been observed in bumblebees. The brain volumes of *Bombus impatiens* workers, belonging to several colonies, were found to vary between 0.25 – 3 mm^3 , a 12 times difference in brain size (Mares et al., 2005). The intraspecific variation in *T. evanescens* brain volume was 5.15 times (Table 1), but since even larger wasps than those in this sample can be reared using larger host eggs, it is possible to create a larger intraspecific and phenotypic variation in brain size.

Trichogramma evanescens brain volume was found to range between 9 and 17 % of body volume, constituting on average 13% percent of total body volume. Compared to other animals (Table 2) these are rather large brain sizes, which could be enabled by their parasitoid life style. Since *Trichogramma*

Table 2. Average brain:body mass ratios of several species of animals.

<i>Species</i>	<i>Brain:body ratio</i>	<i>Source</i>
<i>Mammals</i>		
Humpback whale (<i>Megaptera novaeangliae</i>)	0.016 %	Isler et al., 2008
Giraffe (<i>Giraffa camelopardalis</i>)	0.082 %	Isler et al., 2008
Snow leopard (<i>Uncia uncia</i>)	0.31 %	Isler et al., 2008
Western gorilla (<i>Gorilla gorilla</i>)	0.64 %	Isler et al., 2008
Gray fox (<i>Urocyon cinereoargenteus</i>)	0.99 %	Isler et al., 2008
European Mole (<i>Talpa europaea</i>)	1.2 %	Isler et al., 2008
Short-beaked common dolphin (<i>Delphinus delphis</i>)	1.5 %	Isler et al., 2008
Human (<i>Homo sapiens</i>)	2 %	Roth and Dicke, 2009
Common squirrel monkey (<i>Saimiri sciureus</i>)	3.8 %	Isler et al., 2008
Western harvest mouse (<i>Reithrodontomys megalotis</i>)	4.6 %	Isler et al., 2008
Shrew (family Soricidae)	10 %	Roth and Dicke, 2009
<i>Reptiles</i>		
American alligator (<i>Alligator mississippiensis</i>)	0.0085 %	Wehner et al., 2007
Boa constrictor (<i>Boa constrictor</i>)	0.017 %	Wehner et al., 2007
European pond turtle (<i>Emys orbicularis</i>)	0.10 %	Wehner et al., 2007
Jungle runner (<i>Ameiva</i> sp.)	0.85 %	Wehner et al., 2007
Tropical house gecko (<i>Hemidactylus mabouia</i>)	1.3 %	Wehner et al., 2007
<i>Birds</i>		
Ostrich (<i>Struthio camelus</i>)	0.039 %	Wehner et al., 2007
Wild Turkey (<i>Meleagris gallopavo</i>)	0.12 %	Wehner et al., 2007
Least Sandpiper (<i>Calidris minutilla</i>)	1.7 %	Wehner et al., 2007
Meadow Pipit (<i>Anthus pratensis</i>)	3.3 %	Wehner et al., 2007
Goldcrest (<i>Regulus regulus</i>)	6.6 %	Wehner et al., 2007
<i>Insects</i>		
Bumblebee (<i>Bombus impatiens</i>)	0.4 %	Mares et al., 2005
Leaf cutter ant (<i>Atta colombica</i>)	0.5 %	Seid et al., 2011
Sahara desert ant (<i>Cataglyphis bicolor</i>)	1.2 %	Wehner et al., 2007
Rover ant (<i>Brachymyrmex</i> sp.)	15 %	Seid et al., 2011

evanescens larvae develop in eggs of butterflies and moths and feed on the contents of these eggs, the wasps do not need to provide eggs with all nutrients necessary to complete the development of their offspring, which is why they have to invest relatively little in eggs compared to non-egg parasitoids (Grebennikov, 2008). The wasps might have been able to reach their small body sizes for this reason, but it could also allow them to invest relatively more in their nervous system than other insects or even non-insects. However, the values found in *Trichogramma evanescens* are not the largest relative brain sizes

found in insects. The smallest ant species in the sample of Seid et al. (2011), had brains constituting over 15 % of their body mass. The larger relative brain size of the smallest ants could be caused by their social behaviour, which might require more or larger neurons and therefore a slightly larger brain than the one of a parasitic wasp.

Genotypic and host effects

The insignificant deviations from isometry found for the relationships between brain and body volume for the wasps of GD011 and GD034 (Figure 8) indicate that these wasps show the same relative brain volume independently of their body volume. This finding was unexpected and contradicts the relationship found for GD025, which did deviate significantly from isometry. Although the three regression lines were very different from each other, ranging from insignificant positive allometry to significant negative allometry, there appeared to be no significant effects of the wasps' genotype on the relationship between their body and brain volume. Because no significant difference between the relationships of the three strains was found, the same allometric relationship can be assumed to be present for all *Trichogramma evanescens* strains. A larger sample size is necessary to show if the separate regression lines indeed do not differ from each other.

Host species (Figure 9 a and b) was found not to influence the relationship between brain volume and body volume in *Trichogramma evanescens*. This means that wasps show the same allometric relationship, independently of the species they used as hosts, and that wasps bred on *Ephestia kuehniella* can therefore be compared to wasps emerging from *Mamestra brassicae* eggs. This finding is important for further investigations, since it allows the use of different host species to create a large variety of wasp body sizes, without influencing their relative brain sizes.

Choice of fixative

The original aim of the brain volume measurements was to find an easy measurable body length parameter that predicts brain volume. Since brain size depends on the size of the head capsule, the expectation was that head width would predict brain volume more accurately than the length of the wasps' tibia and body would. However, this was not observed in the fit of the regression lines. Both hind tibia length and body length (Figure 7 a and c) were found to explain brain volume better than head width (Figure 7b). For the correlation with hind tibia length the FMP-treated wasps had an R^2 of 0.89, while R^2 was only 0.82 in the relationship between brain volume and head width. In the GPA-treated wasps, these values were 0.83 and 0.76 respectively. If one would like to estimate brain volume without

using a confocal laser scanning microscope, I would advise to measure hind tibia length, since the tibias are unlikely to shrink as much during fixation as the heads and bodies do, thereby representing a more fixed factor than head width or body length. The lack of changes in tibia length during fixation might also explain why this body length parameter is a better estimator of brain volume than body length and head width, since heads and bodies (unlike tibias) contain a lot of watery tissue capable of shrinking during dehydration, which would consequently increase the amount of variation in the dataset.

From table 1 and Appendix table 3 it is clear that the alcohol-based fixative FMP results in a heavier shrinkage of the tissue than GPA does, as both volumes of brains and heads fixed in FMP only reach 2/3rds of the volumes found in the GPA-treated wasps (largest brain volumes were $1.326 * 10^6 \mu\text{m}^3$ for FMP and $1.953 * 10^6 \mu\text{m}^3$ for GPA, largest head volumes were $2.251 * 10^6 \mu\text{m}^3$ and $3.450 * 10^6 \mu\text{m}^3$ for FMP and GPA respectively). In both samples equally large wasps were present, based on their hind tibia length, and therefore all differences found between the two samples are likely to be caused by the fixative. This effect is also shown by the significant difference between the slopes of the relationships between hind tibia length and brain volume of the GPA- and FMP-treated wasps (Figure 7a). The different slopes show that larger wasps shrink relatively more when treated with FMP than with GPA. This becomes visible because the parameter on the X-axis, hind tibia length, hardly shrinks. A lack of significant difference between the slopes of the relationships between brain volume and head width (Figure 7b) shows that the two fixatives shrink tissue of large wasps in relatively the same amount as small wasp tissue. The fact that the absolute shrinkage is largest with FMP is shown by a difference in the intercept between the two regression lines.

The volume of the head depends on the size of the brain that is inside and therefore an isometric relationship was expected between brain and head volume. However, there was an unexpected, significant difference between the slopes of the regression lines of the two samples. The GPA regression deviates significantly from isometry, whereas the slope obtained using FMP as fixative does not differ significantly from 1, and might be isometric. It appears that the size of the head does not depend completely on the size of the brain inside. The largest wasps have brains that are slightly smaller than expected from their head volume. It is possible that other tissues in the head, such as facial muscles and glandular tissue, increase in a weak positively allometric way, resulting in a head that is slightly larger for the largest wasps than expected from their brain volume alone. The finding that the relationship between brain volume and head volume is isometric when using FMP as fixative, but not when using GPA, clearly reflects differences in the fixatives' properties. As stated before, FMP shrinks the tissues in a

larger amount than GPA does, which could result in a reduced ability to discriminate the relationship from isometry.

It might be best to choose the fixative that shrinks the tissue as little as possible, but figures 6 and 7 show that the R^2 values were highest in the data obtained with FMP as fixative, indicating a higher variability in the dataset in which GPA was used. Although the difference between the two used fixatives is scientifically less interesting, quantifying it could be relevant for further experiments, as the choice of fixative appears to influence the data.

Future research

The results of my MSc thesis provide a good basis for further research on the consequences of differences in brain volumes on insect behaviour, intelligence, longevity and neurology. They allow comparison of brain scaling strategies in other insects and even vertebrates, which is why it would be very interesting to repeat my experiments on other species of minute animals and see if they apply the same brain scaling strategy. I expect that other animals with brains approaching the physical minimum will also show the largest possible brain volume relatively to their body size, which would show as isometric or weak negative allometric relationships.

It is possible that the scaling coefficient is only as large as found in this study in the smallest *Trichogramma evanescens* wasps and decreases by further increases in a wasp's body size. It would be interesting to try to create even larger wasps, using larger host eggs, for example those of the tobacco hornworm *Manduca sexta*, and use a piecewise regression analysis to see if the scaling coefficient is indeed different for the smallest and largest wasps (for an example, see appendix section 'diphasic allometry in small and large wasps'). Another possibility is to look not only at the total brain volume of the wasps, but at relative sizes of the different brain parts. It would be interesting to investigate the relative size changes of the different brain areas as brain size increases, since not all brain parts need to change relatively proportional to each other. These analyses would also allow the creation of a brain atlas of *Trichogramma evanescens*, which could be compared to those that were made for other insect species, such as the honey bee (Brandt et al., 2005; Rybak et al., 2010) or *Nasonia vitripennis* wasps (Haverkamp, 2011).

A necessary step in further investigations of *T. evanescens* brain size is to find out why these brains can be so small and still fully functional. It is possible that the wasps are capable to reduce their neuron size further than other insects can, thereby maintaining neuron numbers and the proportion of connectivity among these neurons and consequently creating a smaller version of the same brain without

compromising performance. This theory can be examined using the same pictures that are needed for studying the relative sizes of the different brain areas or for making a brain atlas. Another possibility that needs to be considered is the relocation of certain brain functions to parts of the wasps' sensory periphery or to the ventral nerve cord in the abdomen. Therefore, it is necessary to look at the size of peripheral sensory systems and nervous tissue in the abdomen, and compare them to other species of insects.

Apart from these neurological studies, my results also allow further behavioural and cognitive research with *Trichogramma evanescens*, for example to investigate their memory storage capacity. The allometric relationships found in this thesis allow a relatively easy estimation of brain volume, based on body length parameters such as the length of the tibia of the hind leg. It would then be interesting to see if wasps with smaller brains have shorter duration of energetically costly memory forms (such as protein synthesis dependent long-term memory (LTM; Mery and Kawecki, 2005) than larger brained wasps, since a small wasp brain might experience too large costs of memory maintenance. Training the wasps on butterfly odours and testing their memory in a Y-tube or two-chamber olfactometer could show that in small wasps memory wanes sooner than in larger wasps. It could also be that small-brained wasps form other, energetically cheaper forms of memory than large-brained wasps, which could be tested using anisomycin that prevents the formation of LTM (Huigens et al., 2009). The small wasps might also suffer higher costs of memory formation than the large brained wasps, possibly showing in a reduced longevity after a learning trial. These experiments also allow a focus on the relative differences in brain sizes, to look into the effects of having a relatively smaller or larger brain than wasps of the same iso-female strain.

Conclusion

The results from this MSc thesis reveal that *Trichogramma evanescens* wasps display a negative allometric relationship between brain volume and body volume that approaches isometry. Brain allometry in *T. evanescens* is described by a scaling coefficient of 0.91, more than twice as large as intraspecific scaling coefficients normally found for insects and vertebrates. This finding indicates that these wasps apply a different brain scaling strategy than larger animals and that larger wasps have only slightly smaller relative brain sizes than the small wasps have. Apparently, there is a rather fixed ratio between brain size and body size in *T. evanescens*, possibly because the ratio is fixed in the wasps' genotypes or is most beneficial for wasps of all body sizes.

Brain size constitutes on average 13% of the total body volume in *T. evanescens*, suggesting a large investment in nervous tissue that could be enabled by the wasps' parasitoid life style. Absolute brain size appeared to vary a lot in these studies, both in phenotypic and intraspecific analyses up to an order of magnitude of 5. Since three iso-female strains, consisting of genotypically nearly identical wasps within each strain, were used it is most likely that this large variation is caused by differences in the available amount of nutrition during the wasps' development instead of by genotypic differences. There was no significant difference in brain allometry between the three iso-female strains, indicating that there is no effect of genotype on brain allometry in *T. evanescens*. Host species was also found not to influence the relationship between brain volume and body volume, thereby allowing different host species to be used in order to create a large variety of wasp body sizes in future research on the effects of (relative) brain size on cognition.

My study clearly shows that *Trichogramma evanescens* wasps, which are among the smallest insects on Earth capable of behavioural and cognitive tasks similar to those of much larger animals, apply a brain scaling strategy that is different from all other insects and vertebrates studied so far. Large *Trichogramma evanescens* wasps have relative brain sizes that are almost the same as those of small wasps, suggesting that a brain volume of about 13% of body volume is the best solution for female wasps of all body sizes and might be fixed in their genotypes. It is possible that the wasps are able to reach their minute brain and body sizes because they could reduce the size of their neurons beyond the minimum of other insect species' neurons. Future investigations should focus on finding out if *T. evanescens*' brain scaling strategy is unique in the Animal Kingdom or if it applies to all tiny eggheads.

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Appendix

Untransformed brain allometry

In addition to figure 6b, the data for *Trichogramma evanescens* brain and body volumes are also shown as untransformed values (Figure 12). This figure clearly shows the weak negative allometry found in this sample as a power function with exponent close to 1. Statistical analyses are difficult to perform for power functions, which is why studies of brain allometry usually show their results based on the natural logarithms of their analysed brain and body sizes. This figure is included because it demonstrates the weak negative allometry in a better understandable way than a linear relationship between transformed values.

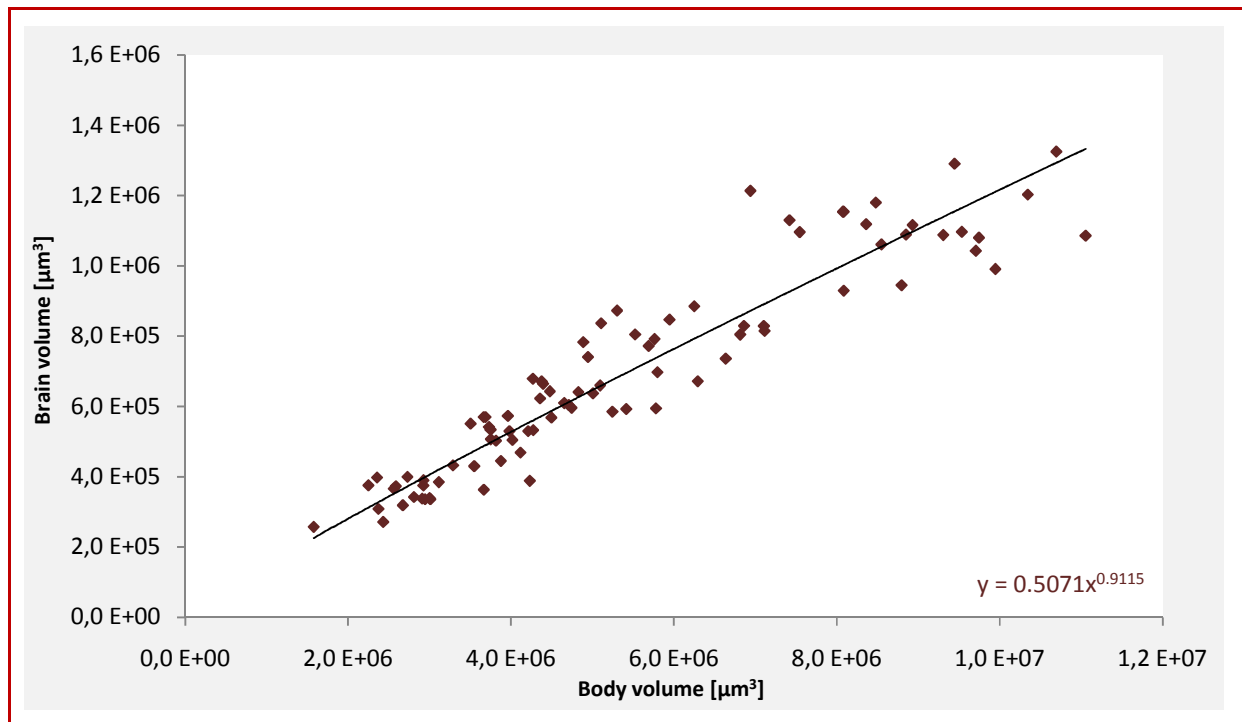


Fig. 12. Scatterplot showing the relationship brain volume and body volume. Wasps were treated with FMP as fixative (n=87).

Diphasic allometry in small and large wasps

It could be that there is no longer an advantage in having a brain as large as possible for the largest wasps in the sample. In that case, diphasic allometry might occur, where the scaling coefficient of allometry is relatively large for the smallest wasps and small for the largest wasps. Similar relationships were found in polymorphic ant species, where the smallest individuals show negative allometric scaling rather close to isometry, and the larger individuals show strong negative allometry (Seid et al., 2011; Wilson, 1953).

The tests for differences in allometric coefficients between small and large wasps were performed in the statistical package R. The method used was a piecewise regression, which tests if a linear regression model consisting of 2 (or more) different lines explains the data significantly better than a one slope model (Crawley, 2007). The point where the first slope of the model changes to the next, is the breakpoint of the model and was found by analysing all possible models (using all unique x-values as potential breakpoints) and choosing the one with the lowest residual standard error. The one slope model was then compared to the two slope model in an ANOVA test to see if the latter fits the data significantly better than the one slope model. The same tests were performed to see if a 3 slope model fits significantly better than the 2 slope model.

Piecewise regressions (Figure 14) showed that a model for all tested *Trichogramma evanescens* wasps with two regression lines and a breakpoint at $7.419 \times 10^6 \mu\text{m}^3$ was significantly better than a model with only one slope (ANOVA, $F_{1,2}=4.064$, $p=0.0207$; 2-slope model – $R^2=0.906$ and $RSE=0.131$; 1-slope model – $R^2=0.899$ and $RSE=0.136$). However, a model with three slopes fitted the data significantly better than the 2-slope model (ANOVA, $F_{1,2}=4.222$, $p=0.0180$) and a 4-slope model was again significantly different from the 3-slope model (ANOVA, $F_{1,2}=4.453$, $p=0.0147$). No further tests were performed for models with more slopes.

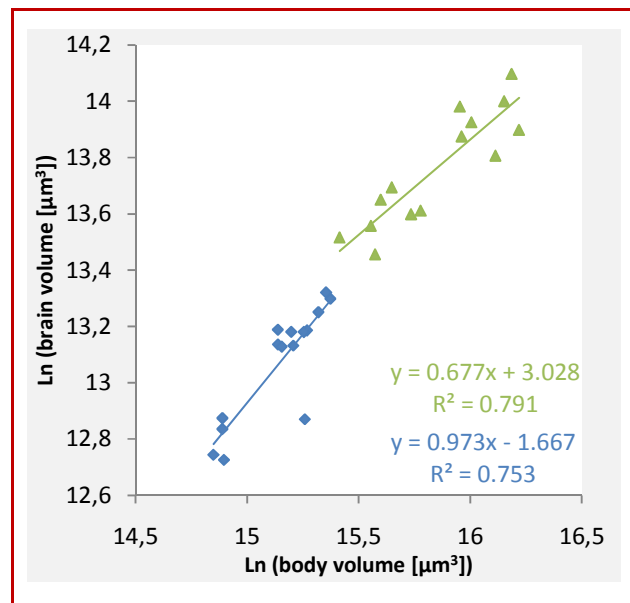


Fig. 13. The 2-slope model in GD011 with a breakpoint at $4.946 \times 10^6 \mu\text{m}^3$.

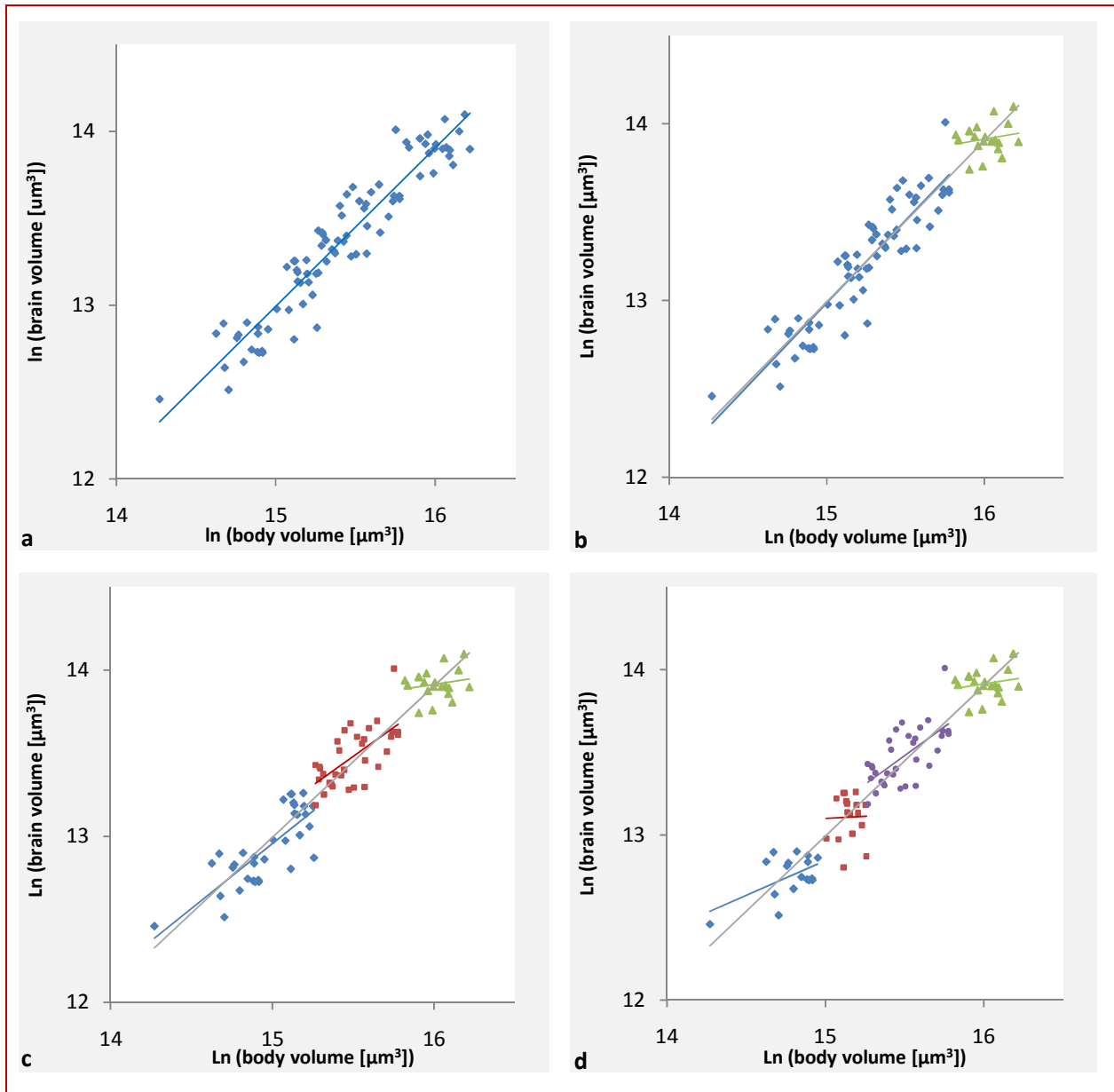


Fig. 14. (a) All wasps, overall scaling coefficient $b = 0.91$ ($R^2 = 0.899$). (b) 2-slope model with breakpoint at $7.419 * 10^6 \mu m^3$, with scaling coefficients $b_{small} = 0.94$ ($R^2 = 0.830$) and $b_{large} = 0.15$ ($R^2 = 0.0384$). (c) 3-slope model with scaling coefficients $b_{small} = 0.96$ ($R^2 = 0.764$), $b_{medium} = 0.85$ ($R^2 = 0.255$) and $b_{large} = 0.48$ ($R^2 = 0.258$) (d) 4-slope model with scaling coefficients $b_{small} = 0.42$ ($R^2 = 0.297$), $b_{small-medium} = 0.057$ ($R^2 = 0.0008$), $b_{medium-large} = 0.70$ ($R^2 = 0.435$) and $b_{large} = 0.15$ ($R^2 = 0.0384$).

When the same was done for the three wasp strains separately, a 2-slope model did not explain the data significantly better than a 1-slope model did in GD025 and GD034 (GD025: ANOVA, $F_{1,2}=3.144$, $p=0.0605$; GD034: ANOVA, $F_{1,2}=2.892$, $p=0.0742$), which could be caused by a too small number of data points per regression slope. In GD011, a 2-slope model with a breakpoint at $4.946 * 10^6 \mu m^3$ (Figure 13) was

significantly better than a 1-slope model (ANOVA, $F_{1,2}=3.456$, $p=0.0473$; 2-slope model: $R^2=0.938$ and $RSE=0.101$; 1-slope model: $R^2=0.923$ and $RSE=0.110$). A 2-slope model for GD011 with a breakpoint closer to the breakpoint found for the combined data (at $9.949 \times 10^6 \mu\text{m}^3$) did not significantly improve the fit to the data compared to the 1-slope model (ANOVA, $F_{1,2}=2.732$, $P=0.0845$). The results from these analyses indicate that there is no clear difference in brain allometry between the small and large wasps in my sample. For future investigations, I would suggest using a larger sample size, covering a larger body size range, to increase the chance of revealing any differences.

Size range of GPA-treated wasps

Shown are the size ranges of the wasps that were treated with the fixative GPA ($n=77$). In this sample no body volumes and body lengths were measured. Absolute volumes and head widths were larger using GPA as fixative than when using FMP (Table 1), but there are no large differences in the orders of magnitudes of variation in absolute volumes and sizes between the two fixatives.

Table 3. Size variation range in *Trichogramma evanescens* wasps treated with GPA ($n=77$) as fixative, showing the size ranges, mean values and the factor of multiplication (largest value / smallest value). The data are shown for the three wasp strains (GD011: $n=26$, GD025: $n=25$, GD034: $n=26$).

	Range	Mean	Factor
<i>Head width [μm]</i>			
GD011	180 – 307	242	1.71
GD025	172 – 328	248	1.91
GD034	204 – 303	248	1.49
Total	172 – 328	246	1.91
<i>Hind tibia length [μm]</i>			
GD011	131 – 235	178	1.79
GD025	139 – 228	181	1.64
GD034	154 – 220	183	1.43
Total	131 – 235	180	1.79
<i>Brain volume [μm^3]</i>			
GD011	386,317 – 1,953,218	1,019,141	5.06
GD025	441,280 – 1,950,609	1,072,675	4.42
GD034	524,103 – 1,706,354	947,742	3.26
Total	386,317 – 1,953,218	1,012,406	5.06
<i>Head volume [μm^3]</i>			
GD011	578,211 – 3,450,107	1,630,648	5.97
GD025	686,239 – 3,022,237	1,726,823	4.40
GD034	886,674 – 2,946,089	1,536,093	3.32
Total	578,211 – 3,450,107	1,629,946	5.97

Host species-dependent size range

Table 4 illustrates the body size range of *Trichogramma evanescens* wasps reared on two different butterfly host species: *Ephestia kuehniella* and *Mamestra brassicae*. From these data, it becomes clear that the smallest wasps emerge from *E. kuehniella* eggs, but that the largest variability of body sizes can be obtained when *M. brassicae* is used as host species. Wasps reared on *M. brassicae* eggs can almost get as small as the smallest wasps emerging from *E. keuniella*.

Table 4. Body size ranges, mean values and the factor of multiplication (largest value / smallest value) for wasps bred on *Ephestia kuehniella* (n=29) and *Mamestra brassicae* (n=58). FMP was used as fixative.

	<i>Ephestia kuehniella</i>			<i>Mamestra brassicae</i>		
	Range	Mean	Factor	Range	Mean	Factor
Body length [μm]	287 – 445	389	1.55	320 – 625	477	1.95
Head width [μm]	162 – 227	199	1.41	168 – 325	248	1.94
Hind tibia length [μm]	114 – 171	147	1.50	126 – 240	195	1.90
Brain volume [μm^3]	257,547 – 679,564	469,528	2.64	336,389 – 1,326,117	806,729	3.94
Head volume [μm^3]	393,102 – 1,099,511	790,783	2.80	583,003 – 2,250,738	1,383,086	3.86
Thorax + abdomen volume [μm^3]	1,183,666 – 3,678,744	2,772,671	3.11	1,662,169 – 9,177,094	4,939,392	5.52
Body volume [μm^3]	1,576,768 – 4,745,397	3,563,454	3.01	2,248,945 – 11,055,407	6,322,479	4.92

Brain volume and body size correlations

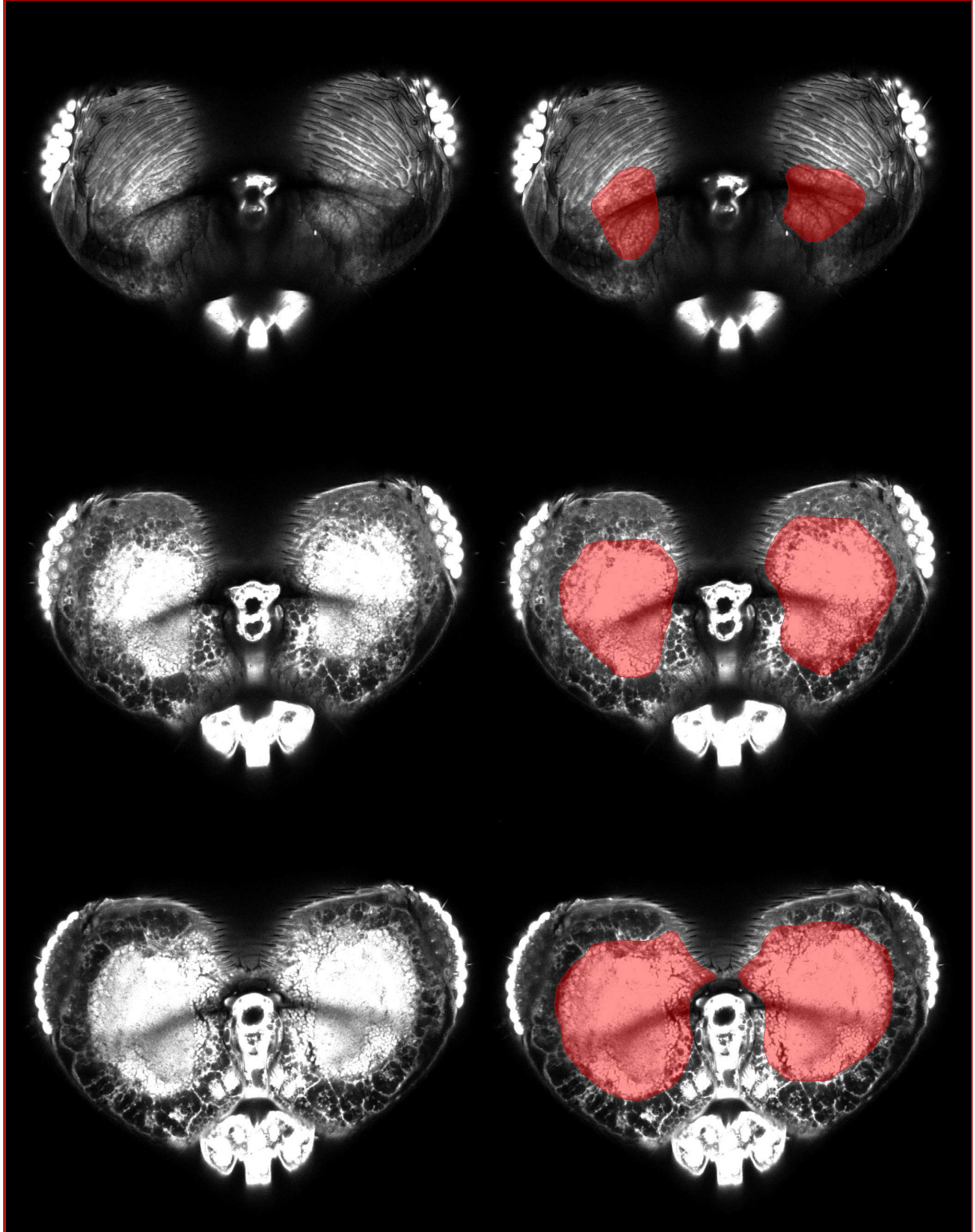
Shown are the correlations between brain volumes and the measured parameters for body size for the entire *Trichogramma evanescens* sample and separately for the three iso-female strains used in these experiments. The measured body size parameters are hind tibia length, head width and body length. The correlations between brain volume, head volume and body volume are also shown. To obtain these correlations, a Spearman correlation was performed (after a Shapiro-Wilk test to test for normality). Least-squares regression was used to obtain the regression line parameters *a* and *b*, in the same way as for the regression lines mentioned in the main part of this thesis.

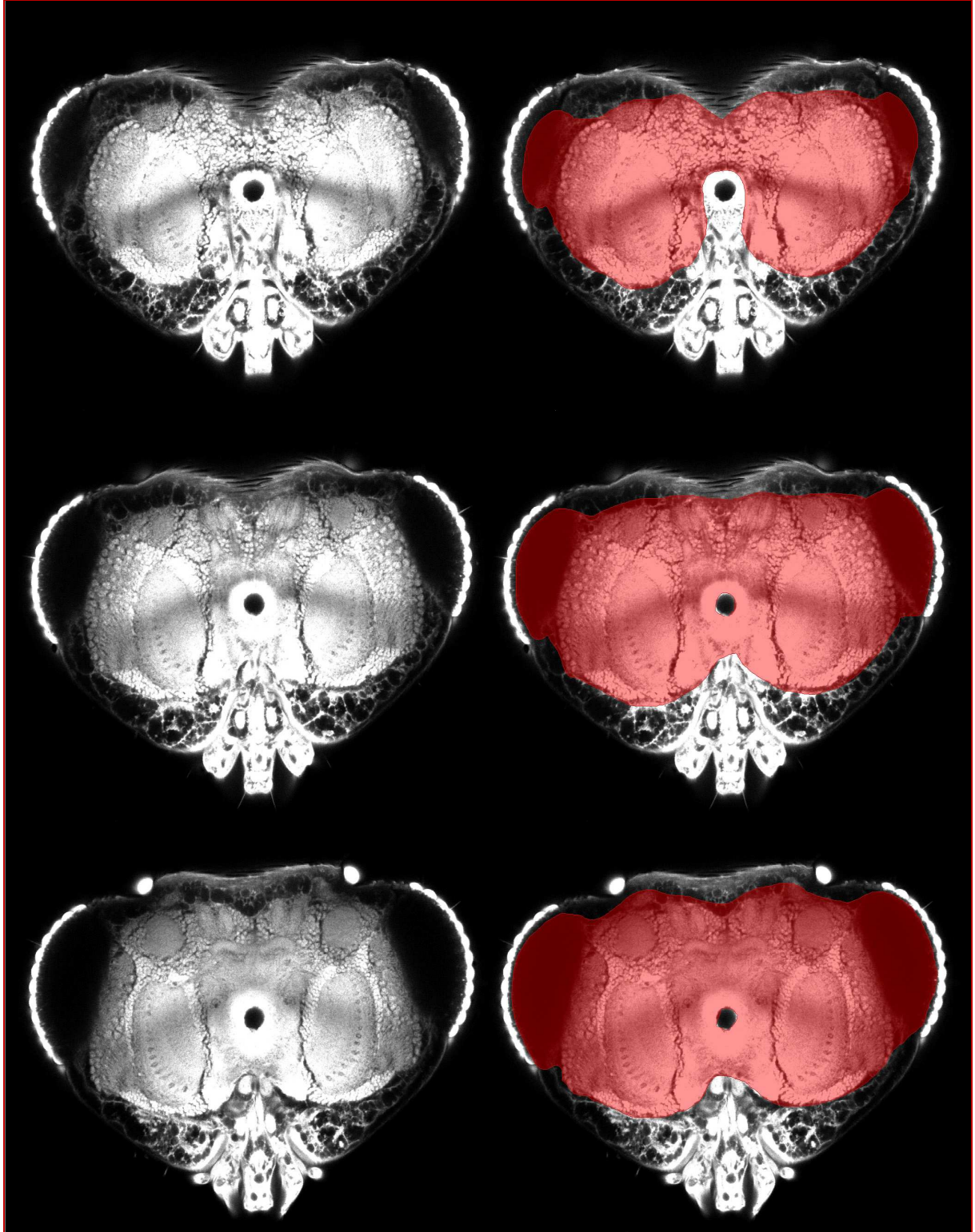
Table 5. Allometric relationship between brain volume and body size parameters for GD011 (n=29), GD025 (n=29) and GD034 (n=29) and for all strains taken together (n=87). Shown are the Spearman correlations (r) with its significance (p_r), regression slope or scaling coefficient (b), intercept (a), number of analysed wasps, regression R^2 and probability that the slope was zero (p_{model}).

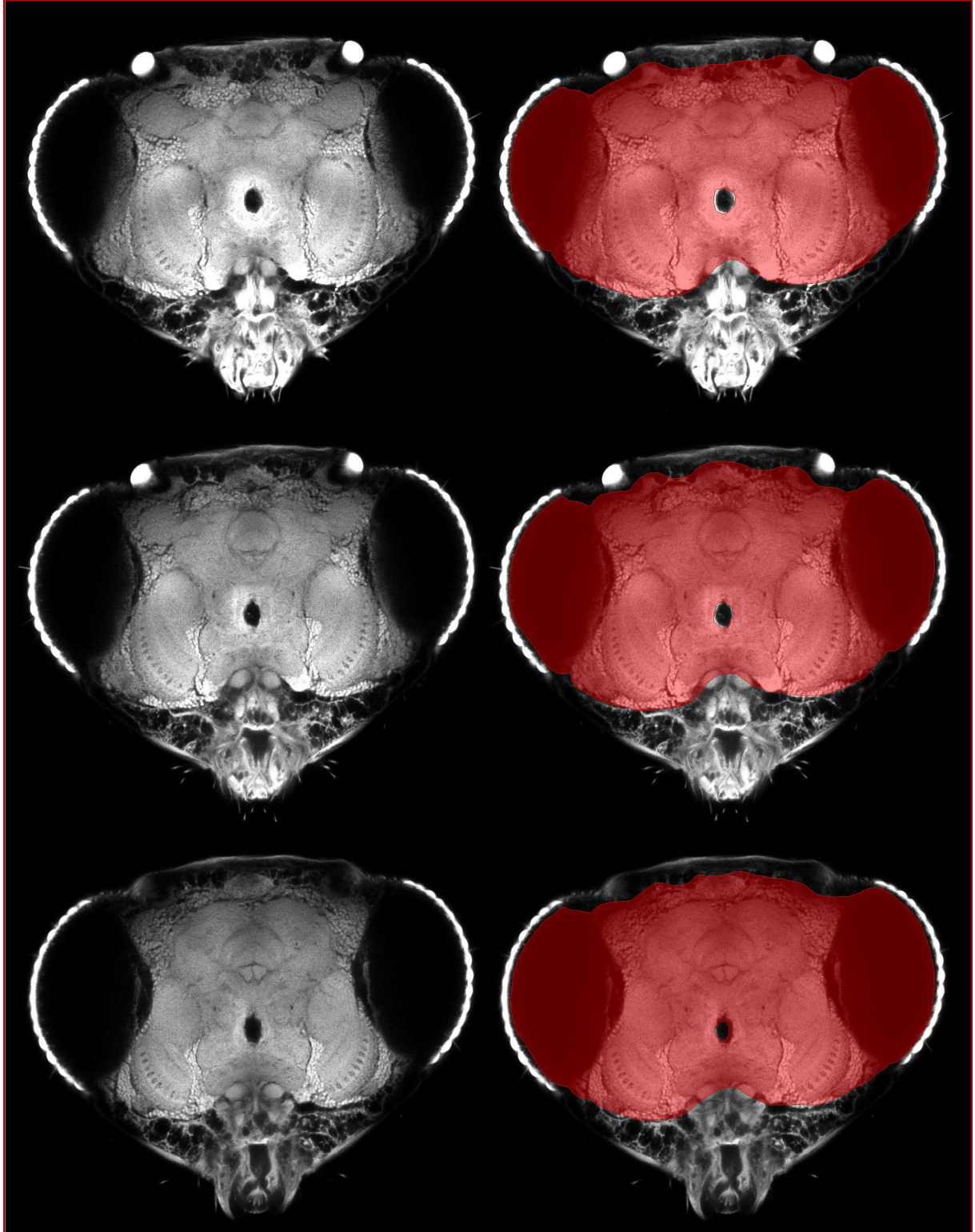
Strain	r	p_r	b	a	R^2	p_{model}
<i>Ln (brain volume [μm^3]) – Ln (hind tibia length [μm]) correlation</i>						
GD011	0.933	<0.001	1.829	3.921	0.911	<0.001
GD025	0.957	<0.001	2.123	2.396	0.929	<0.001
GD034	0.898	<0.001	2.226	1.842	0.857	<0.001
Total	0.939	<0.001	2.042	2.809	0.893	<0.001
<i>Ln (brain volume [μm^3]) – Ln (head width [μm]) correlation</i>						
GD011	0.938	<0.001	2.023	2.356	0.891	<0.001
GD025	0.931	<0.001	2.557	-0.549	0.884	<0.001
GD034	0.854	<0.001	2.772	-1.612	0.754	<0.001
Total	0.907	<0.001	2.333	0.695	0.818	<0.001
<i>Ln (brain volume [μm^3]) – Ln (body length [μm]) correlation</i>						
GD011	0.940	<0.001	2.273	-0.433	0.891	<0.001
GD025	0.926	<0.001	2.068	0.761	0.905	<0.001
GD034	0.857	<0.001	2.623	-2.639	0.743	<0.001
Total	0.909	<0.001	2.253	-0.352	0.834	<0.001
<i>Ln (brain volume [μm^3]) – Ln (head volume [μm^3]) correlation</i>						
GD011	0.975	<0.001	0.984	-0.334	0.980	<0.001
GD025	0.972	<0.001	0.961	0.019	0.986	<0.001
GD034	0.988	<0.001	1.006	-0.605	0.988	<0.001
Total	0.987	<0.001	0.980	-0.248	0.984	<0.001
<i>Ln (brain volume [μm^3]) – Ln (body volume [μm^3]) correlation</i>						
GD011	0.952	<0.001	0.924	-0.911	0.929	<0.001
GD025	0.924	<0.001	0.820	0.729	0.930	<0.001
GD034	0.939	<0.001	1.118	-3.811	0.894	<0.001
Total	0.939	<0.001	0.911	-0.679	0.900	<0.001

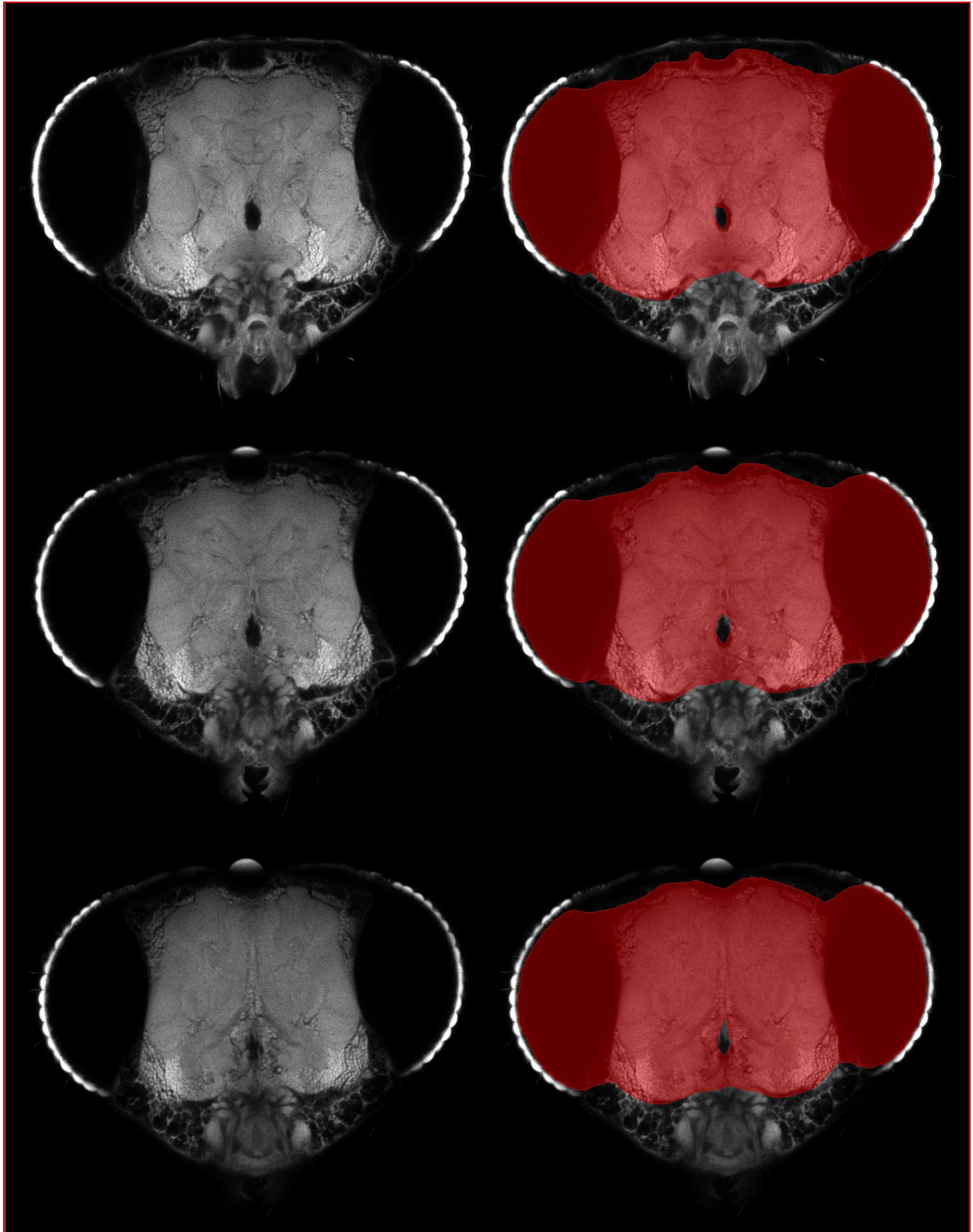
Brain outlines

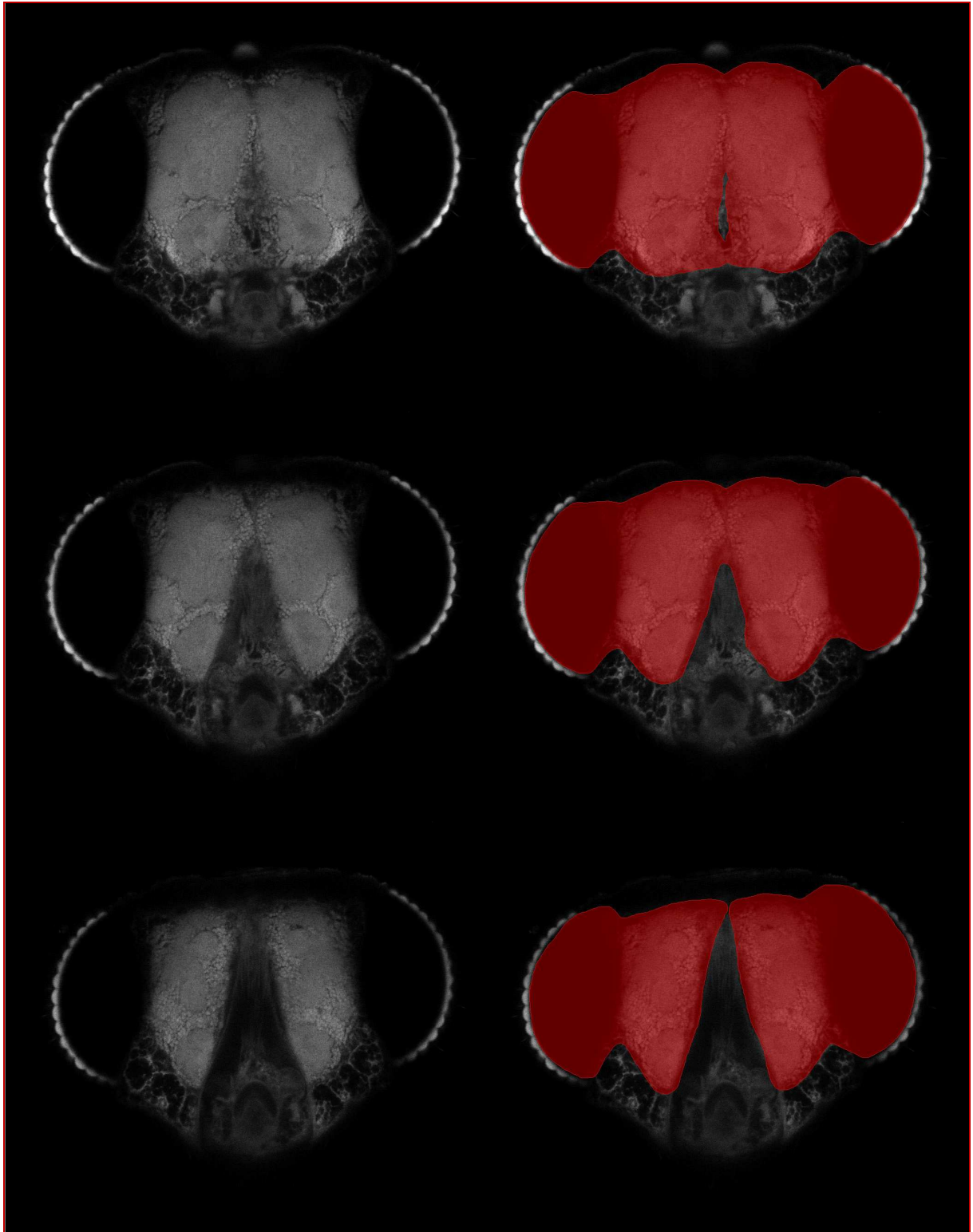
To clarify the definition of brain volume in my study, the following pages show pictures of brain slices (posterior to anterior) and snapshots of brain outlines as drawn on top of those slices in the segmentation editor of Amira 5.3 software. A snapshot was taken every 4 μm in depth of the tissue. The brain outlines were used to create three-dimensional structures in the same software, which were subsequently used to calculate brain volumes. This method was performed for 164 wasps (GPA treatment: n=77; FMP treatment: n=87).











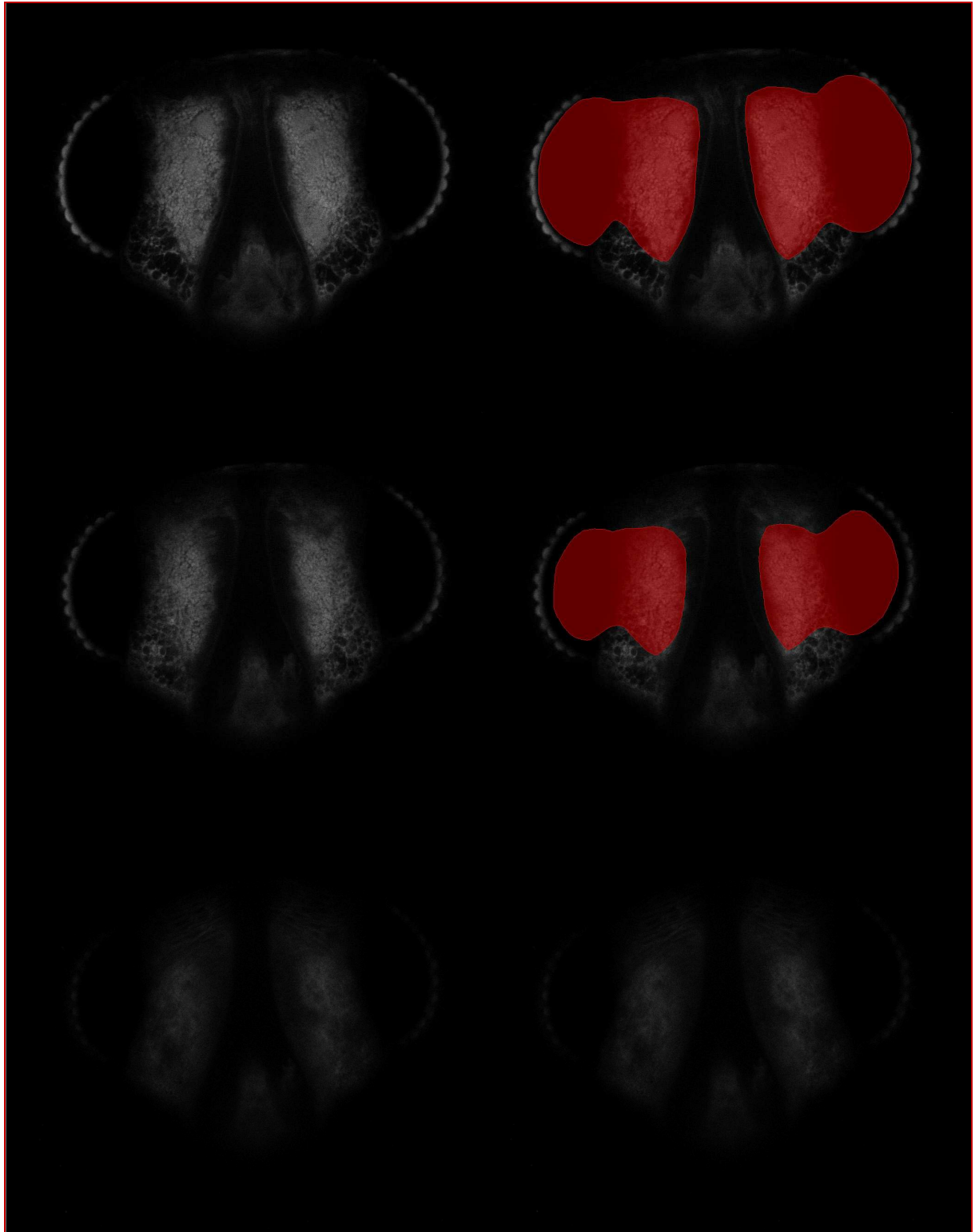


Fig. 15. Brain outlines as drawn in the segmentation editor of Amira 5.3 software. Outlines were used in the same software to compile three-dimensional structures, which were subsequently used to calculate brain volume. Snapshots were taken every 4 μm in depth of the tissue.

