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Fluctuating asymmetry, parasitism and reproductive fitness in two species of gammarid crustacean

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Running page head: Fluctuating asymmetry and parasitism in Gammarus sp.

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Abstract

Fluctuating asymmetry (FA), defined as random deviations from perfect bilateral symmetry, is assumed to reflect developmental instability. FA is predicted to increase in response to environmental stress, including parasite infection. In addition, theory predicts higher FA in sexually selected traits, due to their greater sensitivity to stress. We investigate the relationships between FA, parasitism and reproductive fitness in two species of gammarid crustacean, incorporating both sexual and non-sexual traits. We test the hypotheses that gammarids infected by 1) vertically transmitted Microsporidia will result in higher levels of FA than infection by 2) horizontally transmitted trematodes, due to their increased opportunity to influence development. We found little evidence for a relationship between FA and fecundity in Gammarus spp.; however, the egg diameter for infected female Gammarus duebeni was significantly smaller compared to uninfected female G. duebeni. FA was not correlated with brood size in females, or with sperm number in males. In contrast to our prediction, we report a lower relative FA in response to sexual traits than non-sexual traits. However, FA in sexual traits was found to be higher in males than females, supporting the theory that sexual selection leads to increased FA. Additionally, we report a negative correlation between FA and both trematode (Podocotyle atomon) and PCR positive microsporidian (Nosema granulosis and Dictyocoela duebenum) infections and interpret these results in the context of the parasites' transmission strategies. FA in G. duebeni and G. zaddachi appear to associate with trematode and microsporidian presence, but that reproductive fitness is less altered.

1. Introduction

Fluctuating asymmetry (FA) are random deviations from perfect bilateral symmetry (Van Valen, 1962) and has long been used as an indicator of developmental stability; as it is thought to reflect the inability of the genome to buffer developmental processes against stresses (Zakharov 1992). Hence, FA is often used as a measure of genetic quality in empirical studies (Clarke 1995, Møller & Swaddle 1997), with symmetry assumed to reflect high quality of individuals. However, the response of FAs to stresses appears to be taxon and trait specific (Leung & Forbes 1996), leading to much debate over the relationship between FA and fitness, with levels of FA decreasing with success in sexual competition and fitness in some organisms and traits, but not others (Lens et al. 2002, Tomkins & Simmons 2003, Dongen 2006). Initial meta-analyses reported an overall weak to moderate negative relationship between FA and reproductive fitness (Møller & Thornhill 1998), but have proved controversial, with inconsistency and publication bias in FA studies cited as a likely cause of discrepancies (Clarke 1998, Palmer 1999, Palmer 2000). This has prompted the emergence of publications that have attempted to establish guidelines for the conduct of FA studies (Palmer & Strobeck 1986, Palmer 1994, Lens et al. 2002).

Secondary sexual traits are hypothesised to be particularly sensitive to environmental stress during development and have higher mean FA for their size than morphological traits that are not under sexual selection (Møller & Pomiankowski 1993, Watson & Thornhill 1994). Sexually selected traits are also predicted to show a negative relationship between absolute FA and trait size. Essentially, individuals with larger sexual traits should have lower levels of FA, because they are able to pay the cost of expressing the larger trait, indicating that they are of high genetic quality and perhaps more sexually attractive (Møller & Pomiankowski 1993).

Empirical support for increased FA in sexual traits in comparison to non-sexual traits is again mixed, although few studies have directly compared FA in sexual and non-sexual traits in the same individuals (Bjorksten et al. 2000). Literature surrounding the relationship between FA and measures of reproductive investment is also inconsistent, with few studies having been conducted in invertebrates. Woods et al. (2002) report no relationship between FA in non-sexual traits and egg number in Drosophila melanogaster. Similarly, in male yellow dung flies (Scathophaga stercoraria), no relationship was found between leg FA and sperm size or testis size (Hosken et al. 2003). A negative association between limb FA and ejaculate size was seen in male decorated field crickets (Gryllodes sibilates), but not in the moth Plodia interpunctella (Gage 1998). These examples suggest that multiple sexual and non-sexual traits may be developmentally linked in certain species but without correlation in others.

Multiple parasite groups, including ectoparasites and endoparasites, exert stress and metabolic costs and cause physical changes to the tissues they infect. Due to the associated costs and potential damage to host tissues, infection is predicted to lead to increased FA at all stages of development. The time of infection in the animal's life stage (juvenile vs adult) is also of interest and may have specific

consequences for developmental alterations and specifically FA. This is supported by many empirical studies reporting a positive relationship between prevalence and intensity of infection, and levels of FA (Møller 1996). Alibert et al. (2002) report a positive association between FA in non-sexual traits and infection by two acanthocephalan species in Gammarus pulex. There are several ways in which an association between infection and FA can be viewed (Alibert et al. 2002). Firstly, the metabolic costs of parasite infection may directly lead to developmental instability (Polak 1993). Alternatively, lower quality individuals that experience greater developmental instability may simply be more susceptible to infection (Møller 1996). A correlation between infection and developmental instability may occur due to more asymmetrical individuals living in more stressful environments (Møller & Swaddle 1997). Further, the duration of infection and the life stage of the host may also pose significant factors to the likelihood of resultant FA (Møller 1996). Some parasites are present at fertilisation via vertical transmission, such as infections caused by Pleistophora mulleri (Microsporidia) that can be passed from mother to offspring and may be latent for a given period of time until they emerge at some stage of host development (Bunke et al. 2015).

As crustaceans, gammarids moult throughout their lives in order to grow, and it is not clear whether or not levels of FA are conserved through successive moults in amphipods, with contrasting results in other crustacean species. In the brachyuran crab Hemigrapsus nudus, sign and magnitude of FA were found to persist throughout three successive moults, indicating that FA levels are determined early in life (Chippindale & Palmer 1993). Conversely, in Daphnia magnia, FA varies randomly in sign and magnitude between moults, suggesting that FA reflects recent growth history and that developmental instability may increase with age (Stige et al. 2006). If FA levels are not conserved across moults in Gammarus, the age at which an individual acquires an infection may be less important than in other systems. Whilst it might be expected that microsporidian parasites would have a greater impact on FA due to their presence from the onset of development, this may be counteracted by the differing virulence transmission trade-offs in horizontally and vertically transmitted parasites. As vertically transmitted parasites rely on host reproduction for transmission, virulence levels are generally expected to be lower (Bandi et al. 2001), which may reduce impact on FA levels.

Gammarid crustaceans are host to viral, bacterial, microsporidian, fungal, protistan and metazoan parasites (Bojko & Ovcharenko 2019) and are an ideal study species in which to conduct a comprehensive investigation into the relationships between FA, parasitism and reproductive fitness. Vertically transmitted parasites are common in the Amphipoda (Bojko & Ovcharenko 2019) and can be present from the onset of host development. In contrast, horizontally transmitted parasites can infect the host at any point throughout its life history. Two such species that exhibit vertically and horizontally transmitted infections include Gammarus duebeni and its microsporidian parasites, Nosema granulosis (vertical transmission) and Dictyocoela duebenum (vertical transmission), and Gammarus zaddachi and its trematode parasite Podocotyle atomon (horizontally transmitted). In G. duebeni microsporidian parasites are vertically transmitted from the mother to offspring via the cytoplasm of her eggs (Terry et al. 1998). Male offspring are an evolutionary dead-end for the parasite, because sperm cannot pass on the parasite to the zygote (Dunn et al. 2001); hence it is adaptive for the parasites to feminise hosts,

assuming they are fertile. It may be possible that higher levels of FA are present in infected males that have become feminised, as has been observed in rhizocephalan infected crabs (Grapsus albolineatus) (Li et al. 2002).

We use two gammarid species, G. duebeni and G. zaddachi to investigate the relationships between FA, parasitism, and reproductive fitness. To our knowledge, the full extent of sexual selection has not been well studied in amphipods (Nahavandi et al. 2011). However, we incorporate sexual characteristics, as well as more commonly used morphological traits as a comparison. We test the hypothesis that infection of gammarids by vertically transmitted Microsporidia will result in higher levels of FA than infection by horizontally transmitted trematodes, due to their increased opportunity to influence development. In G. duebeni, we investigate the relationship between FA in non-sexual vs. sexual traits, and measures of fecundity, including sperm numbers in males and egg numbers and diameter in females. We also assess the impact of infection by feminising microsporidian parasites on FA. In G. zaddachi, we examine the relationship between FA in the same (sexual and non-sexual) traits and infection with P. atomon, in both males and females. In contrast to microsporidian infection of G. duebeni, which is present in the embryo, P. atomon prevalence and burden increase with age in G. zaddachi, providing a system to explore how burden and age of infection may influence FA. Overall, this study provides a comparison of the impacts of vertically transmitted and horizontally transmitted parasites on FA levels in two Gammarus spp.

2. Materials and Methods

2.1 Animal collection and husbandry

Gammarus duebeni and G. zaddachi were collected from Budle Bay, Northumberland, U.K. (55°40'N, 1°43'W) during October-November 2010 using a fine mesh net (1mm^2). Gammarids in both stock tanks and experimental pots were maintained in aerated brackish water (15 ppt) at 14℃, 16h light: 8h dark, with rotted sycamore (Acer pseudoplatanus) leaves and algae (Enteromorpha spp.) provided for food and shelter. Both the water and food supplies were replaced daily, to ensure the welfare of the animals. Any gammarids remaining at the end of the study were either maintained in stock tanks or returned to the field.

2.2 Experimental design

2.2.1 Fluctuating asymmetry and fecundity in Gammarus duebeni infected with Microsporidia

To investigate the relationship between FA and fecundity in G. duebeni, 30 males and 30 gravid females were dissected for measurement. Males were isolated in 200ml plastic pots for seven weeks, to allow maximisation of sperm stores before being anaesthetised in carbonated water and dissected for FA measurement (Fig. 1; Table 1) and sperm counts. The FA measurements were made by the same

person using a microscope and graticule throughout the study. To obtain females with eggs in early stages of embryogenesis (stage 1) eggs, 30 pre-copulatory pairs of G. duebeni were isolated, inspected twice daily and separated post-copulation. Gravid females were anaesthetised to allow the new eggs to be flushed from the brood pouch, using a fine jet of water from a hypodermic needle and syringe. The eggs were then counted and the diameter of 10 eggs from each female were measured under an Olympus BH-2 compound microscope. All eggs were then stained with DAPI (4', 6-diamidino-2-phenylindole, diluted 1:500 in 0.2M NaH_2PO_4; a fluorescent stain for DNA) using methods from Kelly et al. (2003), and screened for microsporidian infection to explore relationships between infection, FA and fecundity in females (Fig. 2a).

2.2.2 The relationship between FA and trematode infection in Gammarus zaddachi

To investigate the relationship between FA and trematode infection in G. zaddachi, 60 males and 60 females were anaesthetised and dissected for FA measurements to be taken. Infected/uninfected individuals were identified by examination for the presence of parasite cysts below the cuticle, using a dissecting microscope (Leica S6D) (Fig. 2b). Visual identification of infection was deemed acceptable as it had previously been shown that 84.2% of animals with one or more visible cysts were PCR-positive for P. atomon infection and only one of 58 individuals with no visible cysts was PCR-positive (1.7%) (K. Arundell et al. 2019).

2.3 Dissection and FA measurements

All individuals were anaesthetised, gently blotted dry on tissue paper and weighed. They were placed in a watch glass containing a few drops of Van Harreveld crustacean saline (NaCl, 12g; KCl, 0.4g; $CaCl_2$ $2H_2O$, 1.5g; $MgCl_2$ $6H_2O$, 0.25g; $NaHCO_3$, 0.2g; pH, 7.3–7.4) as specified by Helluy and Thomas (2003) and observed under a dissecting microscope. Watchmaker's forceps and spring loaded scissors were used to decapitate the gammarid, by making a cut between pereons 1 and 2 and coxal plates 1 and 2 (all nomenclature for Gammarus spp. body plan used according to Gledhill et al. (1993) (Fig. 1). The telson was removed by cutting between pereon 7 and epimera 1 and discarded. The pereon and pleon were transferred to Eppendorf tubes and stored in 70% EtOH at -20℃ until require d for measurements to be taken.

A range of measurements were selected, to include both sexual and non-sexual, as well as metrical and meristic traits (Table 1). All measurements were taken at each side of the individual. Non-sexual traits were selected based upon those in which FA had been found to correlate with acanthocephalan infection in a previous study of FA in G. pulex (Alibert et al. 2002). Additional sexual characteristics were also measured to look for a relationship between FA in sexual traits and fecundity. In males, measurements were taken from the genital papillae, which are used to thrust sperm bundles into the brood pouch of the female during mating and the second pereopods. The genital papillae are sexually dimorphic; in males these are enlarged into gnathopods and used to position and hold the female during

copulation (Hume et al. 2005). The number of calceoli on antenna 2 were also counted in male G. duebeni because they are thought to be required for accurate assessment of female quality (Dunn 1998). Measurements were taken from the female's oostegites, which are specialised structures used to form the ventral brood pouch.

All measurements were made at 40X or 100X magnification as appropriate, under an Olympus BH-2 compound microscope. Watchmaker's forceps and fine hypodermic needles were used to dissect and manipulate the tissues as required for measurement. To aid visualisation of the genital papillae, the samples were dehydrated in 500µl 100% EtOH for two hours. The EtOH was then removed and replaced with 500µl cedar wood oil to clear the sample. After another two hours, the tissue was removed from the cedar wood oil and transferred to a microscope slide and viewed at 100X magnification for measurements to be taken.

2.4 Sperm count data collection

Male G. duebeni were dissected for sperm counts as described in Arundell et al. (2014). Briefly, the testes were dissected from the body cavity using spring-loaded scissors, and watchmaker's forceps. The ventral portion of the body was stored with the head in 70% ethanol at -20℃ until required for FA measurements. The testes were transferred to 10µl of distilled water on a cavity slide and ruptured using a fine hypodermic needle. The sample was then washed into an Eppendorf tube using a Gilson pipette and diluted to a final volume of 1ml. The tube was gently vortexed in order to evenly distribute the sperm, before three 10 µl aliquots were pipetted onto a microscope slide and allowed to air-dry. The slides were viewed using an Olympus BH-2 microscope at 40X magnification and the sperm were counted and summed together. Counts from the 3 aliquots were averaged and multiplied by the dilution factor (100X) to give an estimate of the total number of sperm for each male.

Similar measurements could not be made for G. zaddachi because this species did not pair well in laboratory conditions and so reproductive measurements would have been unlikely to be representative.

2.5 Microsporidia screening in amphipod hosts

To determine infection status of the mother, eggs were screened for microsporidian parasites (Kelly et al. 2001, Weedall at al. 2006). Briefly, eggs from each female were collected in separate Eppendorf tubes. A Pasteur pipette was used to add 1ml of 5M HCl to each tube, until the membranes had dissolved – evident due to a colour change from dark grey to bright orange. At this point, the HCl was removed and the eggs washed with distilled water, before being stored in 1ml acetone at -20℃ until required for DAPI screening (Fig. 2a).

When required for visualisation, eggs were transferred to a microscope slide and left to allow excess acetone to evaporate. A cover slip was placed over the eggs and gentle pressure applied, to spread

out the cells of the eggs without destroying them. A Gilson pipette was used to deliver 150µl of DAPI quick mounting solution, which fluoresces in the presence of DNA, to the edge of the cover slide, so that it then covered the eggs by capillary action. Any excess DAPI solution was removed with tissue paper and the edges of the coverslip sealed with clear nail varnish. Slides were stored at 4℃ in the dark and visualised as soon as possible. The slides were viewed at 200X magnification under a Zeiss Axiovert S100 microscope, using a mercury light source and appropriate filters for visualising the DAPI staining (exciter filter BP 450-490; chromatin beam splitter FT510; barrier filter LP520). Infection by N. granulosis or D. duebenum was determined by the presence of parasite DNA in the cytoplasm around the host nucleus.

2.6 Data Analysis and model selection

Unless otherwise stated, all data were analysed using statistical models constructed in R version 3.1.1 (R Development Core Team 2014). All models were initially constructed as maximal models, including all relevant terms and interactions. Models were compared using P-values from the "dropterm" function (MASS library; Venables & Ripley 2002) to determine whether terms significantly improved the fit of the model. Those that didn't were removed in a stepwise fashion until only terms that improved the fit of the model at $P<0.05$ remained.

2.6.1 Testing for directional asymmetry, antisymmetry and measurement error

When testing FA, it is first important to rule out two other forms of symmetry: directional asymmetry (DA) and anti-symmetry (AS). These both occur when one side of a bilateral trait is always larger than the other; in DA one specific side (left or right) is consistently the largest, whereas in AS, the side that is larger varies randomly among individuals (Van Valen 1962). Additionally, due to the often small nature of measurements taken, it is important to ensure that measurement error (ME) is not outside acceptable limits, specifically that differences in between-sides measurements are significantly larger than differences in repeated measurements (Palmer & Strobeck 1986). To test for ME, repeated measurements were carried out on all metrical traits for a subset of 60 individuals. Repeated measures were carried out three times, over the course of three days, without reference to the previous measurements, following the method of Palmer (1994). Significant errors in recording meristic data were ruled out by repeat counting of the first sample for each trait. DA and AS were assessed across the entire data set. Whilst expected distributions for meristic data are less clear than for metrical data, because the meristic traits selected generally differed by relatively large amounts, e.g. 4 or 5 (Palmer 1994), it was deemed adequate to assume they would approximate metrical traits. All traits were subjected to the same analyses by the same person (KLA) for the rest of preliminary testing.

For each trait in each data group, DA was tested for using one-sample t-tests to ensure that the mean of the signed asymmetries did not differ significantly from 0 (Palmer 1994, Alibert et al. 2002). To test for AS, we used IBM SPSS Statistics 20 (IBM, Armonk, NY, USA) to check for any departures from

normality, with Shapiro-Wilk and Kolmogorov-Smirnov tests, as well as outputting skewness and kurtosis estimates (Palmer 1994). Sequential Bonferroni correction was used to correct for multiple tests (Rice 1989). Measurement error was evaluated using a linear mixed effects model (Pinheiro et al. 2013) for each trait, including side as a fixed factor and individual as a random factor. A significant interaction term between side and individual indicated that measurement error was sufficiently minimal to proceed with FA analysis (Palmer 1994).

We checked for any size-dependence of FA using three methods (Alibert et al. 2002, Palmer 1994). 1) To test for within-samples size-dependence, we ran multiple linear models (LMs) of right and left sizes (|R-L|) against both weight of the individual and trait size (R+L/2), using sequential Bonferroni correction when interpreting the outputs. 2) To test for among-samples size-dependence, LMs of log(var(R-L)) vs. mean((R+L)/2) across metrical and meristic traits were constructed. 3) As the tests detected evidence of among-samples size dependence, two different indices of FA were calculated for all further analysis (see Section 5.4.1), FA3 which accounts for mean trait size, and FA1 that does not (Palmer 1994):

FA1 = |R-L|

FA3 = |R-L|/mean(R+L)

Where R = right measurement and L = left measurement

2.6.2 Fluctuating asymmetry and fecundity analysis for Gammarus duebeni and microsporidian infection

To investigate any relationship between FA and egg number or egg diameter, general linear models (LMs) were constructed, including the measure of FA, female weight and infection status as predictor variables. For the egg number model, egg diameter was also included as a predictor variable, to look for a potential trade-off between egg number and egg size. To look for an association between FA and microsporidian infection in females, a generalised linear model (GLM), with binomial error structure, of infection status against FA index was used, also including female weight as a potential predictor variable.

2.6.3 Fluctuating asymmetry analysis for Gammarus zaddachi and trematode infection

To test for a parasitic association between the trematode P. atomon and FA in G. zaddachi, GLMs, with binomial error structure, of infection status against FA index were constructed separately for males and females. Both models also included individual weight as a potential explanatory variable.

2.6.4 Differences in FA between traits

To test for differences in FA between the various traits measured for this study a GLM of FA3 for all data was used, with quasi-Poisson error structure, as data were non-normal and over-dispersed. FA3 was used as this index is corrected for trait size, thus enabling us to look for effects across the entire data set, without bias due to differences in size between G. dubeni and G. zaddachi, or between males and females. Species, sex, data type (metrical vs. meristic) and trait type (sexual vs. non-sexual) were included as factors.

3. Results

3.1 Exploratory data analyses

The mixed model (side x individual) for the replicate measurements showed that the non-directional asymmetry variances (interaction variances from model outputs) were significantly larger than measurement error, for all traits in the subset of 60 individuals tested ($P<0.001$ in each case). The mean variance due to measurement error ranged from 1.0% to 10.2% of the total between-sides variance and it was therefore deemed acceptable to take just one measurement for the rest of the individuals (Palmer 1994). Additionally, it was concluded that these values were sufficiently low to avoid significant bias in subsequent statistical analyses.

We found no evidence for directional asymmetry (DA) in any of the specimens analysed with one-sample t-tests found no significant departures from a mean of 0 across all 40 signed asymmetry distributions tested.

Kolmogorov-Smirnov tests detected significant departures from normality in 1 of 24 metrical traits and 5 of 16 meristic traits, whilst Shapiro-Wilk tests detected significant departures from normality in 3 of 24 metrical and 2 of 16 meristic traits. Skewness and kurtosis were significant for 3 of 24 metrical and 4 of 16 metrical traits. The same distributions were often significant for more than one of the tests, such that overall, 9 distributions (3 metric and 6 meristic) differed significantly from a normal distribution. However, strong antisymmetry (AS) was ruled out as inspection of histograms for these 9 distributions revealed that none were platykurtic. Due to the low number of significant results and because departures from normality were not concentrated across any particular trait or sample, it was concluded that the samples were suitable for FA analysis.

We found little evidence for any within-samples size-dependence of FA, with LMs for both measures of size (individual weight and trait size) across all metrical traits in all samples (48 tests in total) with P-values > 0.05 (following Bonferroni correction). Two of the 32 tests for meristic traits in all samples did indicate significant dependence of FA on trait size - uninfected male G. zaddachi count of spines on pereopod 6 PER ($P<0.05$) and count of flagellum segments on antenna 2 ($P<0.01$) – yet not on individual weight. As type 1 error is a common hazard in multiple testing, we did not deem this to be

significant evidence to justify correcting for within-samples size-dependence. However, we did find strong evidence for among-samples size-dependence across both metrical and meristic traits ($P<0.001$ in both cases). Hence, we accounted for this size-dependence by calculating index FA3, defined as |R-L|/mean(R+L) (Palmer 1994). Palmer and Strobeck (1986) warn that this correction may mask genuine associations with FA, if the factor under investigation is also correlated with size. In this study, it is likely that measures of reproductive potential (egg numbers, and sperm numbers etc.), as well as parasite infection (vertically or horizontally transmitted), may be associated with individual size. Therefore we also used index FA1, defined as |R-L| (Palmer 1994), for comparison in all analyses. Additionally, to look for differences in FA among trait-types, we used subsets of the data to calculate mean FA3 for metrical and meristic, and sexual and non-sexual traits, separately. Number of oostegites in females was found to be exactly equal for the left and right sides in all individuals examined; therefore, this trait was excluded from the analyses.

3.2 Fluctuating asymmetry and fecundity in Gammarus duebeni infected with Microsporidia

We found no relationship between FA and brood size in G. duebeni females, using either index of FA; e.g. FA1 for all traits ($F_{1,27}=0.027$, $P=0.871$), nor FA3 for all traits ($F_{1,27}=0.075$, $P=0.787$). Egg number was only significantly affected by female weight, with larger females producing larger brood sizes ($F_{1,28}=16.888$, $P<0.001$, Adjusted $R^2=0.35$, Fig. 3a). We found no evidence for a trade-off between egg number and size, with egg diameter having no effect on brood size ($F_{1,27}=0.623$, $P=0.437$). Additionally, we found no effect of microsporidian infection on brood size ($F_{1,27}=0.104$, $P=0.749$).

By contrast, we found marginal evidence for a relationship between egg diameter and fluctuating asymmetry, with FA1 for all traits ($F_{1,27}=3.179$, $P=0.086$, Fig. 3b), FA1 for metrical traits ($F_{1,27}=3.742$, $P=0.064$) and FA3 for metrical traits ($F_{1,27}=3.431$, $P=0.075$) all showing non-significant trends for a decrease in egg diameter with increased FA. Additionally, egg diameter was significantly reduced by 9.8% in infected individuals (mean ± S.E. 591.4 ± 8.8µm) in comparison to uninfected individuals (mean ± S.E. 655.2 ± 11.5µm) ($F_{1,28}=6.015$, $P=0.021$, Fig. 4a). However, we found no relationship between female weight and egg size ($F_{1,27}=0.001$, $P=0.979$).

Of the 30 G. duebeni females used in the study, 7 (23% prevalence) were found to be infected with Microsporidia. Due to the low sample size for infected females, individuals infected with N. granulosis and D. duebenum were combined for statistical analysis purposes. The binomial GLM found a significant relationship between infection status and FA3 for all traits ($LRT_1=10.082$, $P=0.001$, Fig. 4b), yet not between infection status and FA1 for all traits ($LRT_1=0.896$, $P=0.343$), indicating that the among-samples size-dependence correction in FA3 is enabling detection of an association that is not apparent from FA1 alone. This highlights the importance of selecting the appropriate index for use in studies of FA. Infection status was not associated with individual weight ($LRT_1=0.438$, $P=0.508$).

In male G. duebeni, we found no relationship between sperm number and either index of FA; e.g. FA1 for all traits ($F_{1,27}=0.557$,$P=0.470$), and FA3 for all traits ($F_{1,27}=0.135$,$P=0.716$). However, sperm number

was significantly associated with male weight, with increased sperm numbers in larger males ($F_{1,28}$=87.081, P<0.001; Fig. 5).

3.3 The Relationship Between FA and Trematode Infection in Gammarus zaddachi

We found no relationship between female G. zaddachi trematode infection and FA1 (LRT_1=0.311, P=0.577) or FA3 (LRT_1=0.890, P=0.346), for all traits. However, when looking at mean FA for non-sexual traits only, we found a significant association between infection and FA1, with infected individuals exhibiting higher levels of FA in non-sexual traits than uninfected individuals (LRT_1=8.752, P=0.003). This relationship was not significant between infection and FA3 for non-sexual traits (LRT_1=1.131, P=0.288). However, infection status in female G. zaddachi was strongly correlated with weight (LRT_1=10.398, P=0.001) and hence size of individual. Infected females (mean ± S.E. 16.2 ± 0.9mg) were on average 21.6% heavier than uninfected females (mean ± S.E. 12.7 ± 0.5mg). It is highlighted by Palmer (1994) that if the factor under investigation is also correlated with size, then the size-dependence correction in the FA3 index may mask genuine associations with FA (Palmer & Strobeck 1986). The observed association between trematode infection and FA1 in non-sexual traits is likely to be real (Fig. 6a).

Similarly, in male G. zaddachi, we found a significant relationship between P. atomon infection and FA1 for all traits (LRT_1=22.756, P<0.001, Fig. 6b), but not between infection and FA3 for all traits (LRT_1=1.668, P=0.197). Again, we found a significant association between infection status and weight (LRT_1=4.065, P=0.044), with infected males (mean ± S.E. 36.1 ± 1.4mg) weighing on average 12.2% more than uninfected males (mean ± S.E. 31.7 ± 2.1mg).

3.4 Differences Between Traits

The GLM for FA3 across the whole data set, found a significant effect of the interaction between trait type and sex on FA3 (F_1=17.466, P<0.001). Non-sexual traits showed higher levels of FA3 than sexual traits in both males and females. FA3 in non-sexual traits did not differ between sexes, whereas FA3 in sexual traits was significantly higher in males than females (Fig. 7). Additionally, we found a significant effect of data type on FA3, with meristic traits (mean ± S.E. 0.0540 ± 0.0024) exhibiting higher levels of FA3 than metrical traits (mean ± S.E. 0.0267 ± 0.0010) (F_1=109.280, P<0.001). Species did not significantly affect levels of FA3; hence G. duebeni and G. zaddachi did not differ in FA levels (F_1=2.57, P =0.11).

4. Discussion

Our study suggests that microsporidian and trematode infections can alter the symmetry of a hosts' physical characteristics, including both sex-linked and non-sex-linked traits. Below we explore the fluctuating asymmetry of the traits affected and the parasites associated with the change.

4.1 Host traits and fluctuating asymmetry

We found little evidence of a relationship between FA and fecundity in Gammarus sp. without disease; with no relationship between FA and brood size (egg number) in females, or sperm number in males. We observed a trend for females with higher levels of FA to produce smaller eggs; but, the linking mechanism is unknown. These findings are consistent with the literature, which finds contradictory results regarding the relationship between FA and reproductive fitness, with FA correlated with reproductive fitness and success in some hosts and associated traits, but not in others (Clarke 1995; Møller & Swaddle 1997; Lens et al. 2002; Dongen 2006). For example, no relationship between leg FA and reproductive fitness or longevity was found in female yellow dung flies (Martin & Hosken 2002). Additionally in male yellow dung flies, no association has been found between leg FA and sperm size or testis size (Hosken et al., 2003). Relationships between FA and reproductive fitness could be specific to the species examined and include multi-factorial reasons for their occurrence.

4.2 Parasitism and fluctuating asymmetry in amphipods

To date, the only other study of FA in amphipods investigated the impact of acanthocephalan infection on FA levels in Gammarus pulex (Alibert et al. 2002). Levels of FA1 found in the present study were comparable to those observed in G. pulex despite differences in the species and range of traits measured. However, their study did not use FA3 and so comparisons between size-corrected measures of FA cannot be drawn. In accord with Alibert et al. (2002), we found compelling evidence for a link between FA and parasitism in gammarids, with increased FA in female G. duebeni infected with Microsporidia, and in both male and female G. zaddachi infected by the trematode P. atomon. This is consistent with the theory that FA reflects developmental instability. Known microsporidian parasites of G. duebeni are vertically transmitted, passing from mother to offspring via the cytoplasm of the egg (Terry et al. 1998). Hence individuals are infected from the onset of development, meaning that the increased levels of FA are likely to reflect a reduction in developmental stability caused by infection. This seems particularly plausible as both N. granulosis and D. duebenum are feminisers (Weedall et al. 2006), converting genetic male offspring into functional phenotypic females, in order to avoid the evolutionary dead end of infecting a male. Sperm are comparatively tiny and rarely contribute to the cytoplasm of the zygote, hence they do not transmit cytoplasmic parasites (Dunn & Smith 2001). Therefore, many phenotypic females will have started life as genotypic males and it is not difficult to

imagine that these conflicting influences may lead to significant developmental instability (Weedall et al. 2006).

The metabolic costs of microsporidian infection may also impact on female reproductive success by reducing investment in reproduction. We found a non-significant trend for a reduction in egg diameter in microsporidian-infected female G. duebeni. However, the sample size for infected females was relatively small (7 of 30 females). Hence this area of research may warrant further study. It is likely that larger eggs are advantageous to reproductive success as they enable provision of more resources for the developing eggs. For example, female Gammarus produce fewer, larger eggs in winter, when conditions are less suitable for reproduction due to temperature and diet (Dunn & McCabe 1995; Sheader 1996). Additionally, trade-offs between egg size and number are stronger in smaller Gammarus minus females. Hence larger females, for which space constraints are less of an issue, maintain egg size with an increase in number, again indicating that larger egg size is preferable (Glazier 2000). In line with previous studies (Terry et al. 1998; Kelly et al. 2003), we found no evidence for an effect of microsporidian infection on egg number or size. The same association was made by Bojko et al. (2018) in Dikerogammarus haemobaphes infected with Cucumispora ornata, where the sample size was in the hundreds of individuals examined.

In contrast to microsporidian infection, P. atomon infection increases in prevalence and intensity in larger and hence older gammarids in wild populations; most likely due to increased exposure over time. Therefore, it might be expected that the increased FA in parasitised individuals reflects an increased susceptibility to infection in individuals that experienced higher levels of developmental stability. Crustaceans are particularly interesting for studies of asymmetry because they moult throughout life in order to grow larger. The degree to which FA is conserved throughout successive moults is variable between crustacean species. For example, Chippindale and Palmer (1993) report a persistence in both sign and magnitude of FA throughout three successive moults in the brachyuran crab Hemigrapsus nudus, indicating that FA levels are determined early in life. Conversely, in Daphnia magna, FA was found to vary randomly in sign and magnitude between moults, suggesting that FA reflects recent growth history and that developmental instability may increase with age (Stige et al. 2006). It is not known whether symmetry is conserved across moults for gammarid species and we highlight this as an interesting area for future research. If levels of FA fluctuate between moults, then trematode infection has the potential to increase FA over an individual's life span.

4.3 Concluding remarks and future considerations

In this study, parasitism has been shown to have specific negative impacts on naturally symmetrical traits in amphipods, for both vertically transmitted Microsporidia and horizontally transmitted trematode parasites acquired later in development. Our data suggest that FA can be influenced by parasitism; however, fecundity does not seem to be impacted significantly. This may reflect the parasites need to transmit and utilise the host as well as maintain the host population density and size for continued

transmission. For vertically transmitted Microsporidia, their primary method involves a breeding host, therefor the parasite may be evolutionary predisposed to avoid impairing the breeding capability of the host to benefit its own transmission.

Our data reveal that parasitism impacts traits linked with asymmetry, including those traits directly involved in reproduction as well as additional morphological traits. Novel associations with disease in amphipods, including viral, bacterial and other infections (Bojko & Ovcharenko 2019), have paved the way to conduct further assessment of asymmetry linked with parasitism and the additional discovery of diseases in amphipods and their study as model systems to explore FA could provide intriguing relationships between specific parasite taxa and the likelihood for parasite-associated host developmental alterations.

Acknowledgments

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Figures and Tables

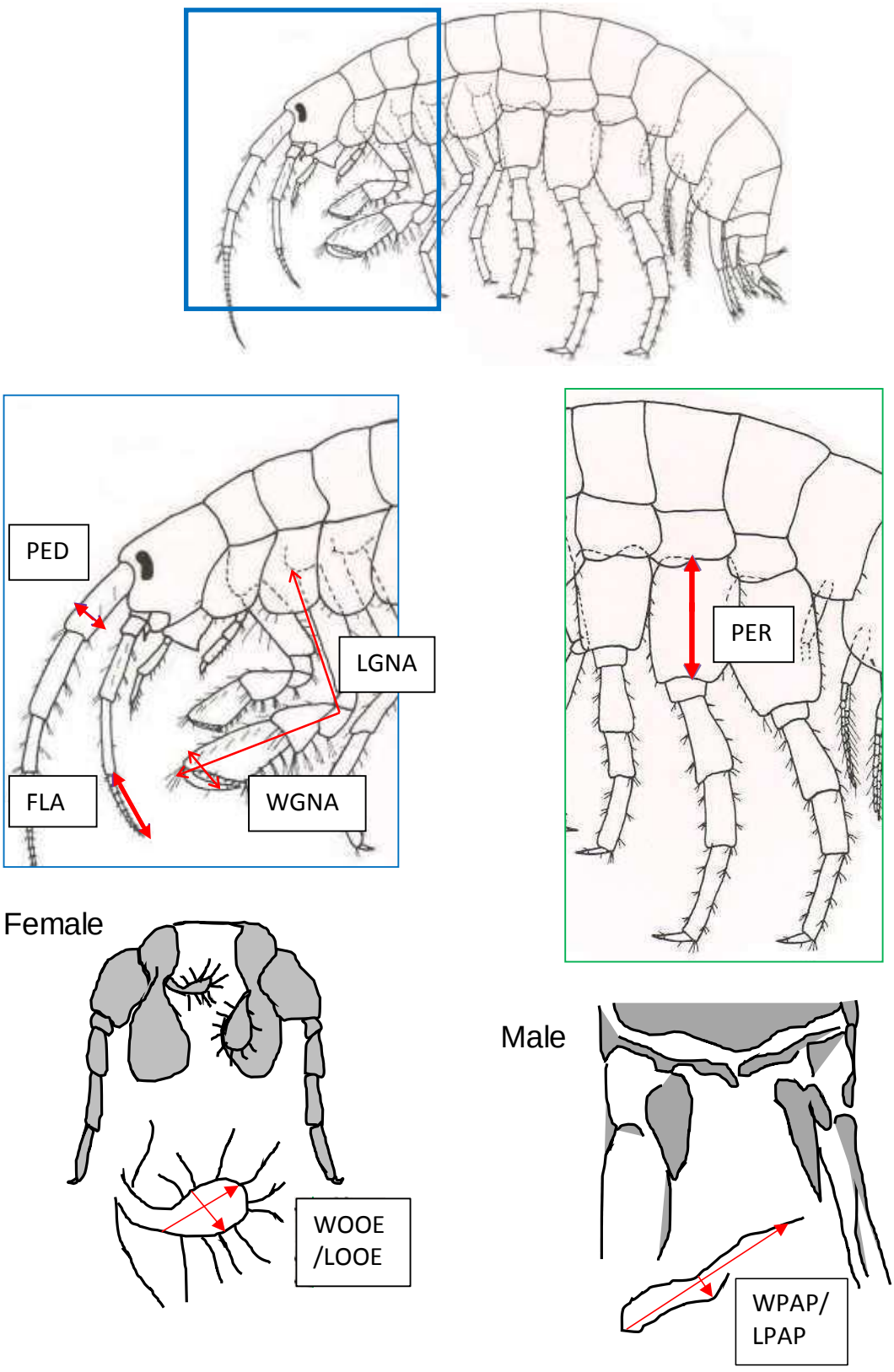

Figure 1. Measurements for FA Calculations; Solid arrows indicate metrical measurements and dashed arrows indicate regions from which meristic counts were taken; Purple arrows indicate non-sexual and

red arrows sexual traits. Diagrams of male and female sexual appendages are reproduced from Gledhill et al. (1993). See Table 1 for definitions of abbreviations.

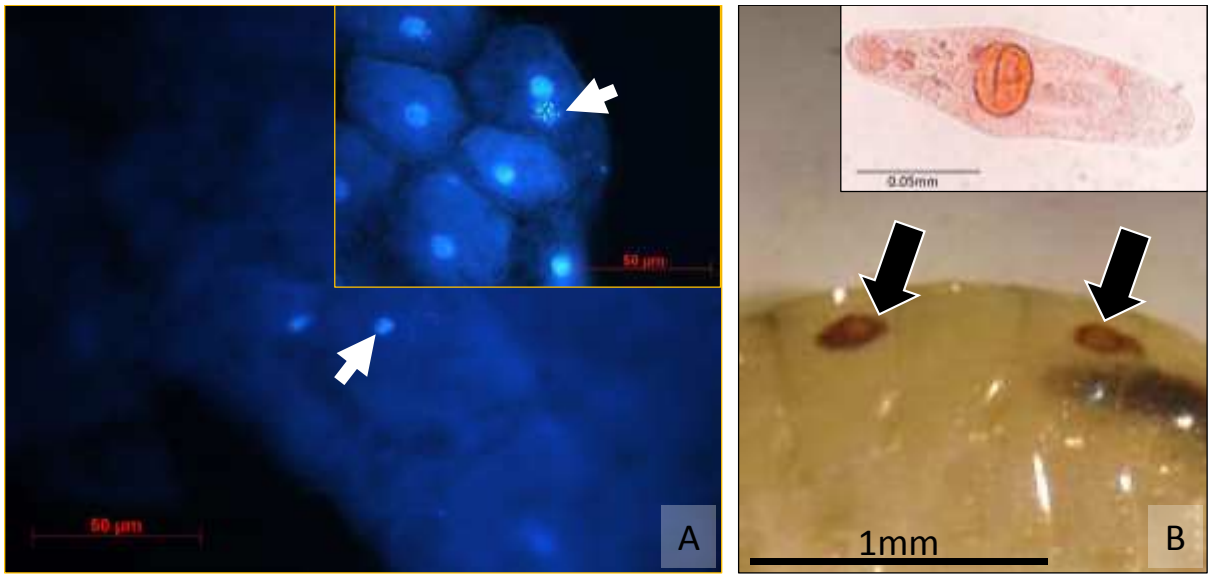

Figure 2: DAPI stained oocytes with and without Microsporidia present in Gammarus duebeni and Podocotyle atomon present in the body cavity of Gammarus zaddachi. A) Tissue with light microsporidian infection. The white arrow indicates a cell nucleus. In the inset image the white arrow identifies Microsporidia present in the cytoplasm of an oocyte. B) Two trematodes encysted in the body cavity of its host. A biopsied specimen is shown in the inset.

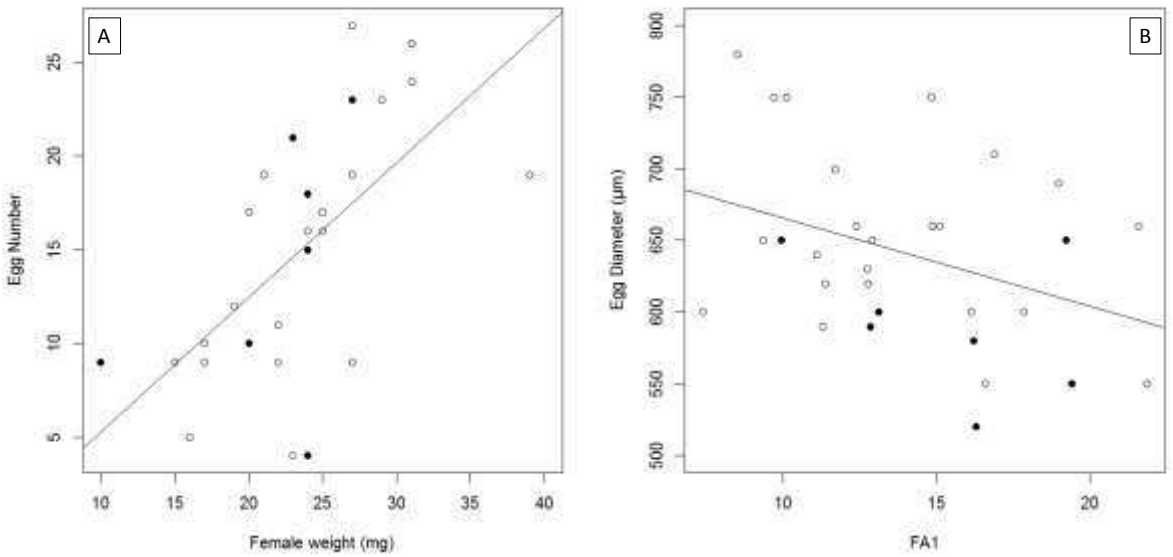

Figure 3. The relationships between a) egg number and female weight (mg); and b) egg diameter (µm) and FA1 (for all traits) in Gammarus duebeni. Individualds determined to be infected with Microsporidia are presented as black circles. Uninfected individuals are presented as clear circles. FA1 = |R-L| (Where R = right measurement and L = left measurement).

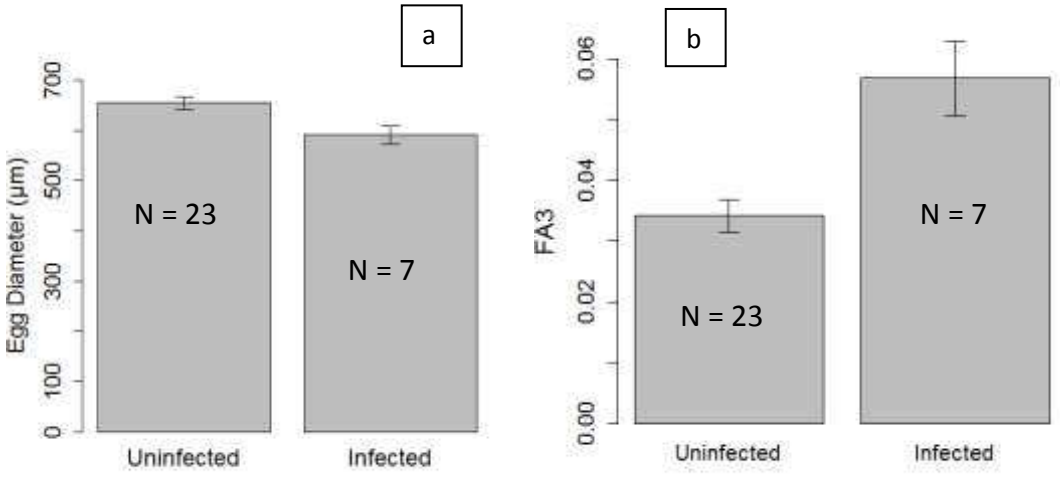

Figure 4. The impact of microsporidian infection expressed on mean a) egg diameter; and b) FA3 (across all traits) in female Gammarus duebeni, with ± 1 S.E. bars.

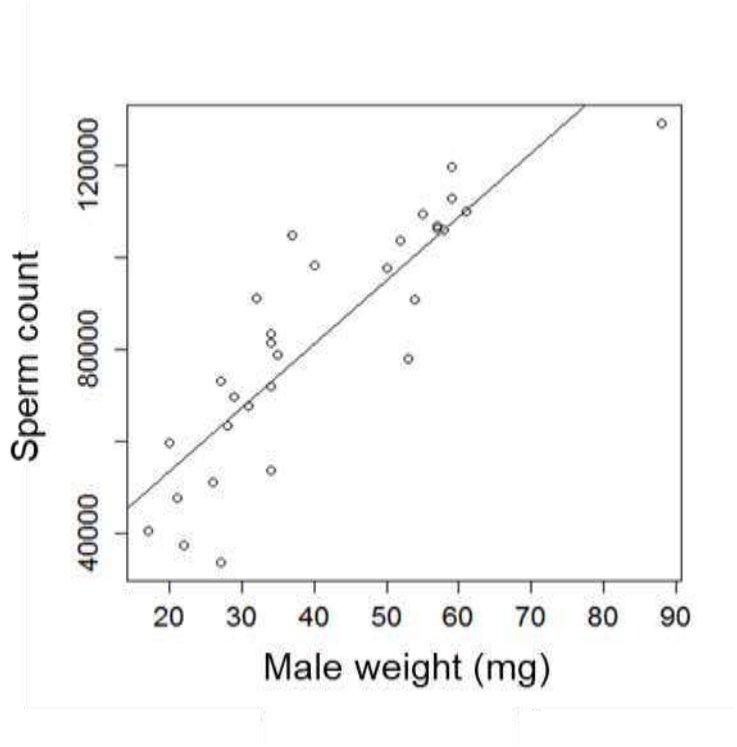

Figure 5. The relationship between weight and sperm number in male Gammarus duebeni.

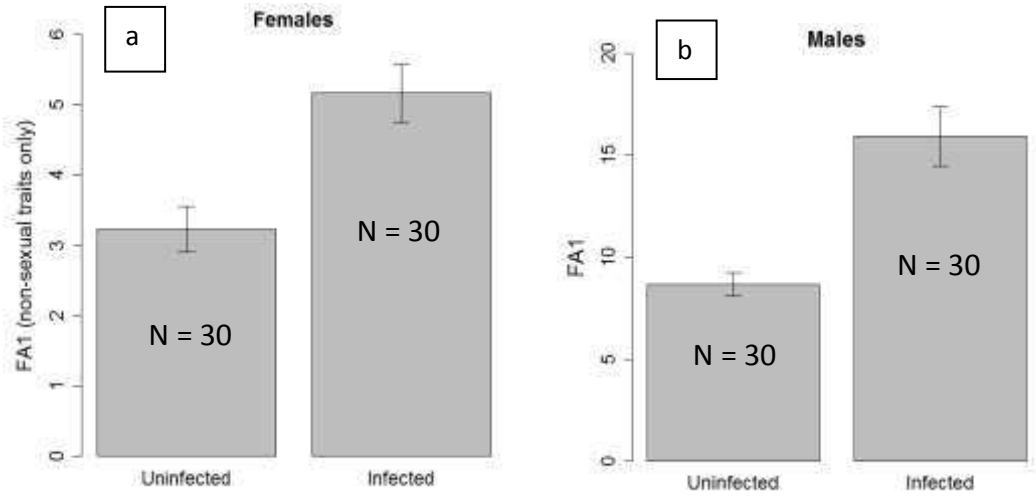

Figure 6. The relationship between P. atomon infection status and: a) FA1 for non-sexual traits in female G. zaddachi; b) FA1 for all traits in male G. zaddachi, with ± 1 S.E. bars. FA1 = |R-L| (Where R = right measurement and L = left measurement).

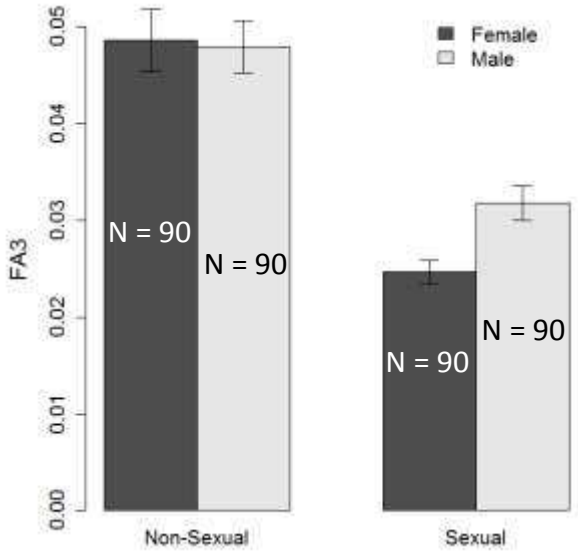

Figure 7. Mean FA3 in non-sexual and sexual traits, for males and females of both G. duebeni and G. zaddachi, with ± 1 S.E. bars.

Trait	Measurement	Abbreviations
Non-sexual: male and females		
Metrical	Width of antenna 1 at the widest part of peduncle article 1	PED
Meristic	Count of flagellum segments on antenna 2	FLA
	Count of spines on the posterior basis of pereopod 6 (walking leg 4)	PER
Sexual: males		
Metrical	Width of pereopod 2 (gnathopod 2) at its widest point	WGNA
	Length of pereopod 2 (gnathopod 2)	LGNA
	Width of tip of genital papillae at widest point	WPAP
	Length of genital papillae	LPAP
Meristic	Count of calceoli on antenna 2*	CAL
Sexual: females		
Metrical	Width at the widest point of primary oostegite (found on coxal plate 2) at widest point	WOOE
	Length of bulbous section of primary oostegite	LOOE
Meristic	Count of hairs on primary oostegite	HOOE
	Count of oostegites	NOOE

Table 1. Fluctuating Asymmetry measurements and abbreviations used for gammarid amphipods. All measurements taken in both G. duebeni and G. zaddachi, except for the number of calceoli, which were counted in G. duebeni males only (*). Abbreviations refer to the anatomical diagrams in Figure 1 that

were measured for both species throughout the study. See Figure 1 for a diagrammatic representation of the measurements.