

# Metamorphosis offsets the link between larval stress, adult asymmetry and individual quality

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## Summary

1. It is poorly understood which traits translate larval stressors into adult fitness in animals where larval and adult stages are separated by metamorphosis. Although fluctuating asymmetry (FA) is often assumed to do so, especially in insects the relationship between larval stress, adult FA and individual quality is often absent. One suggested hypothesis for this is the higher mortality of low quality (hence more asymmetric) animals during metamorphosis (i.e. developmental selection hypothesis).

2. Here we test this hypothesis and also propose and test an alternative hypothesis where metamorphosis is stressful but not lethal and increases FA of all animals up to a certain level (i.e. stressful metamorphosis hypothesis).

3. We manipulated larval stress (food stress and pesticide stress) and measured FA before and after metamorphosis in the damselfly *Coenagrion puella*. Additionally, we assessed the relationship between FA and individual quality variables measured at metamorphosis (age, mass and two immune variables: phenoloxidase (PO) and haemocyte number).

4. Before metamorphosis, FA reflected the combination of food and pesticide stress and was negatively related with mass and both immune variables after metamorphosis. These patterns were, however, offset after metamorphosis. Low mortality, not linked to FA during metamorphosis, indicates that developmental selection cannot explain this. Instead, the strong increase in FA up to equal levels across treatments during metamorphosis supports the stressful metamorphosis hypothesis.

5. Taken together, the developmental stage in which FA is measured may critically determine the reliability of FA as an indicator of stress and of individual quality in insects.

**Key-words:** complex life cycle, damselflies, fluctuating asymmetry, immune function

## Introduction

Many animals have a complex life cycle (CLC) with a discrete larval and adult stage, often separated by metamorphosis (Moran 1994). One challenge for life-history theory is to understand whether and how stressors in the larval stage affect adult fitness in such animals. Although it is typically assumed these effects are entirely mediated through age and size at metamorphosis (Roff 2002), recent studies suggest this may be only partly true (e.g. De Block & Stoks 2005; Stoks, De Block & McPeck 2006a). Therefore, we need studies identifying which other adult traits that may affect adult fitness are shaped by larval stressors and which are not. One

well-studied trait that may bridge the life-history transition is fluctuating asymmetry (FA) (i.e. random deviations from perfect bilateral symmetry). There is, however, a long standing debate about the existence and strength of links between larval stress, adult FA and individual quality (Leary & Allendorf 1989; Møller 1997b, 2006; Leung, Knopper & Mineau 2002). Particularly in insects larval stress does often not shape FA in the adult stage (e.g. Bjørksten *et al.* 2000a; Floate & Fox 2000; Antipin & Imasheva 2001; Woods *et al.* 2002; Bourguet *et al.* 2004; Da Silva, Mendes & Lomonaco 2004; Hoffmann *et al.* 2005; Jones *et al.* 2005; for exceptions see Clarke & McKenzie 1992; Hardersen & Wratten 1998; Hardersen & Frampton 1999; Chapman & Goulson 2000; Stoks 2001).

Several explanations have been proposed to settle the controversy of the equivocal relationships between environmental

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stress and FA (reviewed by Van Dongen 2006): differential mortality (i.e. asymmetrical individuals are the first to die at high environmental stress), trait functionality (i.e. more functional traits are more robust against developmental alterations, therefore, exhibit less asymmetry), interactions between stressors (e.g. the effect of a focal stressor is overruled by a baseline stress level), and low statistical power. A specific case of differential mortality – known as the developmental selection hypothesis (Møller 1997a) – may underlie the absence of a relationship between environmental stress and FA in animals with a CLC. This hypothesis states that too asymmetric individuals do not survive to the next developmental stage due to low developmental stability. If many larvae with high FA die during metamorphosis, this may offset the link between larval stress and adult FA.

Because animals under stress frequently survive metamorphosis, we here suggest another plausible explanation for the equivocal relationship between larval stress and adult FA, the stressful metamorphosis hypothesis. As metamorphosis by itself may be stressful (Heyland & Moroz 2006; Menon & Roman 2007) it may considerably increase FA levels thereby potentially overruling effects of all previous stressors. Note this is a specific case of the above-mentioned interactions between stressors. This uncoupling of larval stress and adult FA will be especially effective when animals are capable of keeping an increasing FA levels during metamorphosis under a certain level above which adult fitness would be too much compromised. This would equalize adult FA at this level across larval stress treatments during metamorphosis. Because no study so far directly evaluated the relationship between larval stress and FA before and after metamorphosis, there are no data available to directly evaluate this hypothesis.

Besides a potential indicator of stress between groups of animals that experience different stress levels, FA is also believed to reflect individual quality within such groups (i.e. for a given level of stress, individuals with a higher capacity to buffer stress will be more symmetrical) (Møller 1999, 2006). However, this relationship is also equivocal (e.g. Leung & Forbes 1997). We suggest that the stressful metamorphosis hypothesis may also explain part of these negative results. If, irrespective of individual quality, all animals eventually end up at the same FA level during metamorphosis, then FA levels after metamorphosis will no longer reflect individual quality. This would not erase differences between low and high quality individuals; these differences would merely become apparent in other variables. Compared to high quality animals, low quality animals are likely to have more problems keeping FA below a certain level, and as a result may pay more in terms of not optimizing other traits such as investment in immune function and energy storage. In the case of equalizing of FA levels during metamorphosis, FA measured before metamorphosis should better reflect individual quality compared to FA measured after metamorphosis. Note that the latter pattern is no direct prediction of the developmental selection hypothesis. This hypothesis assumes that only the better quality individuals survive metamorphosis, but within the group of survivors the link between individual quality and FA may persist.

Here, we explicitly evaluate whether metamorphosis erases the link between larval stress and adult FA, and whether this pattern can be explained by the developmental selection hypothesis or the stressful metamorphosis hypothesis. Under the developmental selection hypothesis, we expect during metamorphosis considerable mortality biased toward individuals with a high larval FA. Under the stressful metamorphosis hypothesis we expect an increase of FA during metamorphosis up to a certain level. In both scenarios we expect equal adult FA levels across groups that experienced different larval stress levels.

We tested these hypotheses by manipulating stress levels during the larval stage and relating the stress levels with FA estimates measured before and after metamorphosis in the damselfly *Coenagrion puella*. We used two stressors: transient food deprivation and subsequent exposure to an insecticide. In another publication on this experiment, we showed that these stressors negatively affected mass before metamorphosis, age at metamorphosis and two immune variables [activity of phenoloxidase (PO) and haemocyte numbers] at metamorphosis (Campero *et al.* 2008). These variables reflect individual quality and are tightly linked with fitness in damselflies (Leung & Forbes 1997; Sokolovska, Rowe & Johansson 2000; Rolff & Siva-Jothy 2004). To test whether FA before metamorphosis will be a more reliable indicator of individual quality than FA after metamorphosis, we related FA before and after metamorphosis with these quality variables.

## Methods

### COLLECTION OF ADULTS AND LARVAE REARING

Eggs were obtained from ten females collected in Overijse (Belgium) that oviposited on wet filter paper. Freshly-hatched larvae were randomly allocated in groups of 30 to plastic containers (20 × 15 × 10 cm) filled with aerated, dechlorinated tap water. Containers were placed at 23 °C with a photoperiod of 16L : 8D. When the larvae were 3-weeks-old, they were placed individually in cups filled with 100 mL aerated tap water. Larvae were fed *ad libitum* twice a day with *Artemia salina* nauplii until they reached the F-3 instar (F-0 being the final instar).

### EXPERIMENTAL SETUP

We set up a 2 (starvation stress) × 2 (pesticide stress) full factorial design. Previously starved and non-starved larvae were exposed to two levels of endosulfan (present/absent). Larvae entered into the experiment when they moulted into the F-3 instar and the first FA-leg measurement (see below) was taken (hereafter called pre-experimental FA). At this moment, half of the larvae were starved for 7 days while the rest of the larvae were fed two live chironomid larvae every day. Immediately after the starvation period, we randomly split each starvation group into both pesticide treatments. During this period, all larvae received two chironomid larvae every day. This period ended when a larva stopped feeding to prepare for metamorphosis and the second FA-leg measurement was taken at this point ('before metamorphosis FA'). The final FA measurement for the legs was taken 24 h after the individuals emerged as adults ('after metamorphosis FA'). At this stage, wing-FA measurements were also taken. The experiment stopped when all animals had metamorphosed (c. 2 months after the treatments started).

The insecticide used as a stressor was endosulfan. For this organochlorine insecticide, concentrations higher than 100 p.p.b. have been found in surface waters in Belgium (Steurbaut *et al.* 2003). Powdered pure endosulfan (Sigma Aldrich®, Bornem Belgium; Pestanal class, purity > 98.9%) was first dissolved in ethanol, then diluted in distilled water to prepare stock solutions of 50 000 p.p.b. and kept in darkness at 4 °C. A sub-lethal concentration of 30 p.p.b. was used because a pilot experiment showed that higher concentrations (i.e. 50 p.p.b.) proved to be lethal in 96 h for half of the individuals. Controls (i.e. individuals not exposed to endosulfan) had an identical volume of ethanol added into their cups as individuals exposed to endosulfan. Pesticide and control solutions were renewed every 7 days in the respective cups. Analysis of the pesticide samples revealed that the actual starting concentration was 31.7 p.p.b., with a degradation rate of *c.* 70% after 7 days. Oxygen, temperature and pH were measured every 2 weeks. Oxygen concentrations (mean =  $9.06 \pm 0.88$  mg L<sup>-1</sup>; 82% saturation), temperature (mean =  $23 \pm 1$  °C) and pH (mean =  $7.1 \pm 0.2$ ) were not dependent upon treatments (all  $P > 0.13$ ).

#### FLUCTUATING ASYMMETRY (FA)

Fluctuating asymmetry estimates were based on three independent measurements of both left and right femurs of all three pairs of legs at the different experimental periods (i.e. pre-experimental, before and after metamorphosis). Additionally, after metamorphosis, three independent measurements of the hind wings were also taken (i.e. from the point where the first antenodal crossvein reaches the wing margin to the wing apex). As some of the individuals did not fully extend their wings at emergence or fell into the water before being removed from the cups, wing-FA was only obtained for 64 out of 99 emerged individuals. Images for the measurements were obtained using a stereomicroscope coupled to a digital camera and analyzed with IMAGE PRO PLUS® v5.0.

FA estimates were calculated through mixed model analyses with restricted maximum-likelihood parameter estimation to distinguish real asymmetry from measurement error following Van Dongen, Lens & Molenberghs (1999). The relative measurement error estimates were 9- to 10-fold smaller than the FA estimates, indicating that the analyses had enough power to detect real FA. This procedure also allowed us to test for directional asymmetry, where the degree of asymmetry is biased in one direction, and anti-symmetry, which has a bimodal distribution due to under-representation of symmetrical individuals. Both directional asymmetry and anti-symmetry can have a genetic base and therefore do not exclusively reflect development stress (Palmer & Strobeck 1992). As neither directional asymmetry (all  $P > 0.05$ ) nor anti-symmetry (all kurtosis > 1.00) was detected, we considered all estimates to reflect FA.

As FA may be biased upwards by trait size, a size-corrected FA estimate was used throughout the analyses. The size-correction was done by calculating the percentage of FA in relation to the length of each leg for each individual (see Tomkins 1999). The results of the analyses were similar using size-corrected FA estimates or raw FA-estimates. Therefore, we only report the results using the size-corrected FA estimates.

#### IMMUNE VARIABLES, MASS AND AGE AT METAMORPHOSIS

We measured immune variables, mass and age at metamorphosis as described in Campero *et al.* (2008). Immune variables were measured

following the procedure described in Stoks *et al.* (2006b). Briefly, we extracted haemolymph by perfusing the thorax of each adult with 300 µL of ice-cold PBS (Phosphate Buffered Saline: 0.15 M NaCl and 10 mM Na<sub>2</sub>HPO<sub>4</sub> · 2H<sub>2</sub>O; pH adjusted to 7.4 with orthophosphoric acid). The haemolymph extract was collected in ice-cold 1.5 mL microcentrifuge tubes and was aliquoted for assaying PO activity and haemocyte counting. We used PBS instead of cacodylate buffer (see Stoks *et al.* 2006b) because samples were analyzed immediately after extracting the haemolymph. Cacodylate buffer allows for longer storage time of the samples, which was unnecessary in our case.

To assay PO activity, the samples were frozen and then centrifuged at 2688 g at 4 °C for 15 min to separate the debris. After centrifugation, 100 µL of the supernatant was mixed with 40 µL of PBS and 60 µL of L-DOPA (10 mM 3,4 dihydroxy-L-phenylalanine, purity ≥ 99% Fluka®) in a 96-well plate. The reaction was allowed to proceed at 30 °C for 45 min and the absorbance was measured every 20 s at 490 nm with a Bio-Rad Benchmark Plus® (Nazareth, Belgium) multi-plate reader. Enzyme activity was measured as the slope of the reaction curve during the linear phase ( $V_{max}$ ). Every sample was run in duplicate and the mean of both readings was taken as the value of that sample in the analysis.

For haemocyte counting, 20 µL of the haemolymph sample was placed into a well of a multi-well microscope slide, stained with 5 µL of ethidium bromide (2.5 mM) and left to dry in a dark box overnight. Afterwards, from each well three images were taken at random positions using a fluorescence microscope (Olympus® B × 51 Aartselaar, Belgium) at a magnification of 400×. Haemocytes were counted on the images with the image analyzer software IMAGE PRO PLUS® v5.0. The mean of the three counts was taken as the value of that sample in the analysis.

#### STATISTICAL ANALYSES

FA of the pre-experimental period showed no difference among treatment groups (all  $P > 0.2$ ) indicating that randomization of the individuals among treatments was successful. To test whether FA reflects larval stress before and after metamorphosis, we ran the hierarchical repeated-measures ANOVA with food stress and pesticide stress as the between-subject variables, and leg and period as hierarchical within-subject variables. The lower order repeated-measures factor was leg (three for each individual) and the higher order repeated-measures factor was period (before and after metamorphosis). This way we took into account that the three leg-FA estimates belong to one individual and that the same set of legs of each individual was measured before and after metamorphosis. Note that degrees of freedom reflect data points rather than individuals (Von Ende 2001).

To evaluate the developmental selection hypothesis, we checked for mortality during metamorphosis, and tested whether FA before metamorphosis was higher in individuals that did not survive metamorphosis compared to those that did survive. To evaluate the stressful metamorphosis hypothesis, we tested for a significant increase in FA (positive effect of period) up to a certain level (generating a stress treatment × period interaction) in the hierarchical repeated-measures ANOVA. As FA estimates did not vary between sexes (all  $P > 0.4$ ), sex was not included in the final model.

Finally, we tested whether FA reflects individual quality (i.e. whether FA was linked with PO, haemocyte numbers, mass and age at emergence). Therefore, we ran separate ANCOVAs per period for each quality variable including starvation stress and pesticide stress as independent variables and the size-corrected FA estimates (before or after metamorphosis) as covariates. Significant effects of the FA covariates would indicate a link between FA and quality.

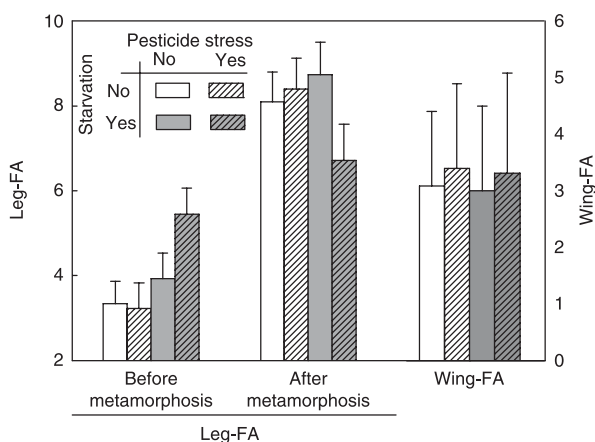
When analyzing PO and haemocyte numbers, we also included mass at metamorphosis in the model as both immune variables may strongly depend upon mass.

## Results

Only 1% ( $n = 5$ ) of the animals died during metamorphosis. Size-corrected leg-FA before metamorphosis did not differ between animals that did not die and animals that died during metamorphosis (survived animals: 1.34, SE = 0.087; dead animals: 1.39, SE = 0.025;  $t$ -test = 1.21,  $df = 97$ ,  $P = 0.21$ ).

Size-corrected leg-FA considerably increased during metamorphosis (period:  $F_{1,540} = 68.76$ ,  $P < 0.001$ ) (Fig. 1). Further, size-corrected leg-FA depended on the starvation stress  $\times$  pesticide stress  $\times$  period interaction ( $F_{1,540} = 4.20$ ,  $P = 0.041$ ). Separate analyses per period only showed a significant starvation stress  $\times$  pesticide stress interaction before metamorphosis ( $F_{1,270} = 4.356$ ,  $P = 0.038$ ). This interaction reflected the fact that size-corrected leg-FA was only higher than controls (i.e. animals without starvation stress and without pesticide stress) when starvation stress and pesticide stress were combined (Fig. 1). After metamorphosis, size-corrected leg-FA levels of the different stress treatments did not differ (all  $P > 0.2$ ). Wing-FA also did not differ significantly among treatments (all  $P > 0.4$ ).

Size-corrected leg-FA estimates before metamorphosis were negatively related with PO (slope =  $-0.099$ , SE = 0.041,  $P = 0.020$ ), haemocyte numbers (slope =  $-2.71$ , SE = 0.95,  $P = 0.005$ ) and mass at metamorphosis (slope =  $-0.0005$ , SE = 0.0001,  $P = 0.016$ ), but not with age at metamorphosis ( $P = 0.324$ ) (Fig. 2). However, none of these variables were related to size-corrected leg-FA after metamorphosis,  $h$  (all  $P > 0.3$ ) or wing-FA (all  $P > 0.5$ ) (Fig. 2).



**Fig. 1.** Mean (+ 1SE) size-corrected leg and wing fluctuating asymmetry (FA) estimates as a function of starvation stress and pesticide stress. Each mean is the per treatment-group average of the individual combined leg-FA estimates as generated by the hierarchical repeated-measures ANOVA. Leg-FA estimates before and after metamorphosis periods are shown separately.

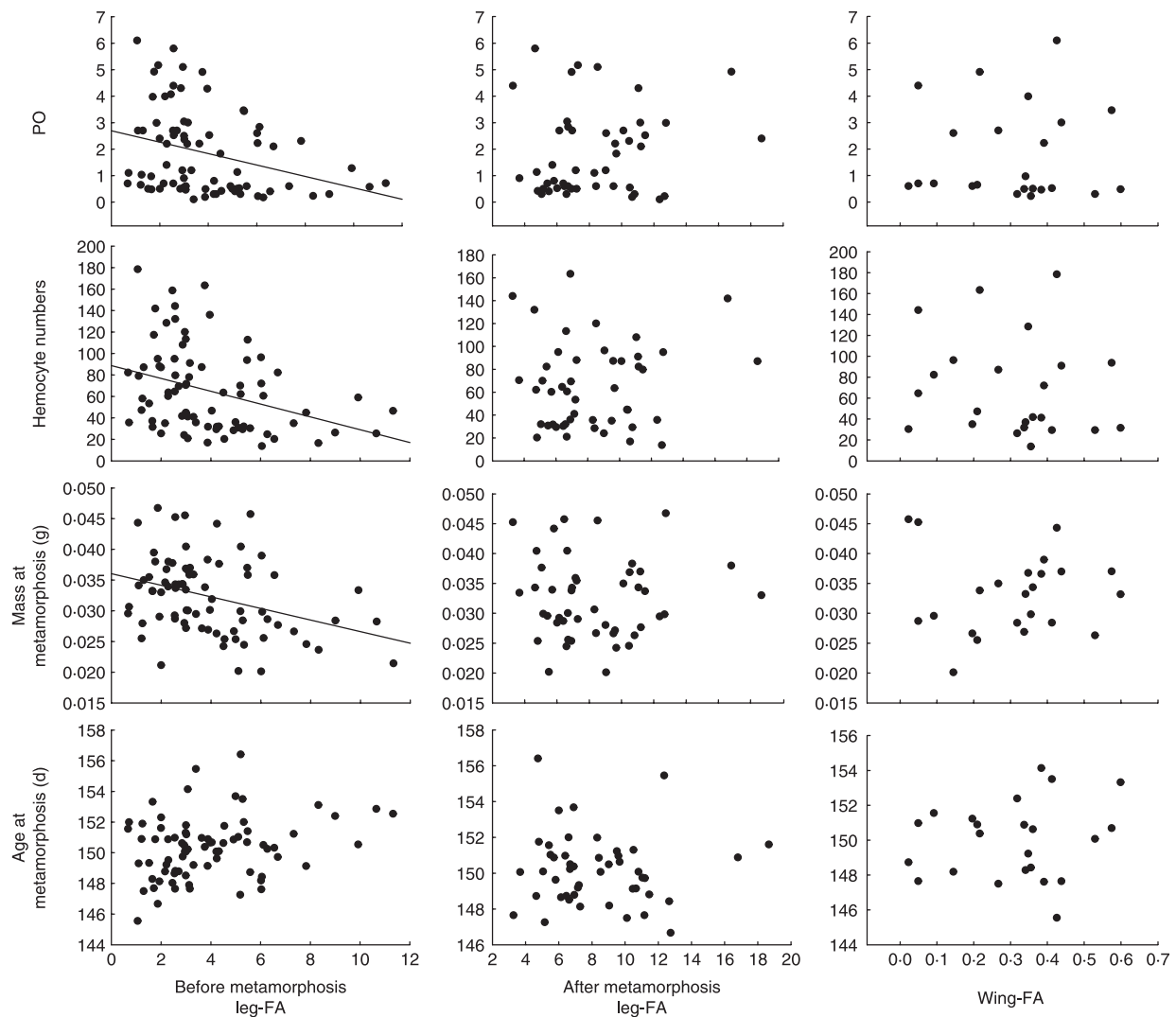
## Discussion

As expected, metamorphosis erased the link between larval stress and adult FA. At the end of the larval stage and prior to metamorphosis, larvae that had experienced a combination of starvation stress and pesticide stress were more asymmetric. Increased asymmetry in response to pollution or food level has been shown before in a broad range of taxa (e.g. Eeva *et al.* 2000; Hardersen 2000). However, our study shows no relationship between larval stress and FA levels measured after metamorphosis. This is consistent with the often observed pattern of no relationship between adult FA and the stress endured during the larval stage in insects (e.g. Bjorksten *et al.* 2000a; Floate & Fox 2000; Antipin & Imasheva 2001; Woods *et al.* 2002; Bourguet *et al.* 2004; Da Silva *et al.* 2004; Hoffmann *et al.* 2005; Jones *et al.* 2005; for exceptions see Clarke & McKenzie 1992; Hardersen & Wratten 1998; Hardersen & Frampton 1999; Chapman & Goulson 2000; Stoks 2001).

It could be argued that the lack of a relationship between larval stress and adult FA (i.e. FA after metamorphosis) is simply due to the fact that the terrestrial adult was removed from the stressor. However, leg and wing sizes (and, therefore, also their FA patterns) are fixed at metamorphosis in damselflies and therefore no further correction is possible. Moreover, larval stressors did negatively influence life-history and immune variables after metamorphosis (i.e. increased age at emergence, decreased PO and haemocyte numbers, Campero *et al.* 2008). Furthermore, the disappearance of the link between larval stress and leg-FA after metamorphosis cannot be explained by differential mortality during metamorphosis (developmental selection hypothesis, Møller 1997a) or too low statistical power, as only 1% of the animals died during metamorphosis and mortality did not depend upon leg-FA before metamorphosis. For wing-FA caution should be exerted when interpreting the results. Animals with not fully enclosed wings could not be included, as this would not only reduce sample size (i.e. power) but also could bias the results if mortality or weakness (animals that fell into the water) was not random with regard to wing-FA.

We found strong support for the stressful metamorphosis hypothesis to explain the mismatch between larval stress and adult FA. In line with this hypothesis, leg-FA increased during metamorphosis in all treatment groups, thereby eventually equalizing FA levels across treatments after metamorphosis. Previous studies indicated that metamorphosis is a stressful event (Heyland & Moroz 2006; Menon & Roman 2007). For instance, it has been shown that oxidative stress considerably increases during amphibian metamorphosis (Menon & Roman 2007). The fact that the increased leg-FA levels after metamorphosis were equal across stress treatment groups supports the notion that animals increased their FA levels up to a certain level above which adult fitness would be too much impaired (see below).

A possible mechanism how animals could equalize FA levels is found under the adaptive decoupling hypothesis (Moran 1994). Although never referred to in the context of FA, the latter hypothesis states that in species with a CLC



**Fig. 2.** Co-variation between leg and wing fluctuating asymmetry (FA) estimates and PO, haemocyte numbers, mass and age at metamorphosis. Size-corrected leg-FA estimates before and after metamorphoses are shown separately. Only significant regression lines are shown (see text).

metamorphosis may uncouple the larval and adult stage to ensure independent adaptation to the environment. Adaptive decoupling may be the result of either compartmentalisation (i.e. adult structures are formed from cell lineages that are undifferentiated until metamorphosis, as in several holometabolous insects like *Drosophila*) and/or remodelling and reorganization (i.e. adult structures are modified from larval structures, as in amphibians and some hemimetabolous insects) (reviewed by Moran 1994). Adaptive decoupling could equalize the adult FA patterns if during metamorphosis a remodelling or reorganization of the legs occurs whereby the increase in FA is kept below a certain level through a higher re-allocation of resources to stabilizing processes in more asymmetric animals to restrict the increase in asymmetry. Potentially, this re-allocation mechanism is triggered only at a certain larval FA level as this correction is likely to be costly and needs re-allocation of energy away from other processes such as, for example, energy storage.

Decoupling larval stress and adult leg-FA during metamorphosis and maintaining FA levels below some maximum level may be adaptive as leg asymmetry may reduce prey-capture capacity and therefore nutritional condition, as has been shown in the hemipteran *Callicorixa vulnerata* (Nosil & Reimchen 2001) and the dungfly *Scathophaga stercoraria* (Swaddle 1997). Additionally, individuals with more asymmetric legs can be more vulnerable to predation as shown by Swaddle (1997) in *Musca domestica*. Decoupling larval stress and adult FA may also be beneficial if the latter is coupled with lifetime mating success, as is the case for wing-FA in *C. puella* (Harvey & Walsh 1993).

In line with the prediction that before metamorphosis FA should better reflect individual quality, before metamorphosis leg-FA had a strong, negative relationship with variables reflecting individual quality (i.e. both immune variables and mass at metamorphosis), while after metamorphosis leg-FA and wing-FA did not. With regard to immune variables, a

review by Møller (2006) showed that, overall, there is a positive relationship between parasite load or disease susceptibility and adult FA. However, individuals with a CLC were underrepresented in this study (around 25%). For example, of the six combinations between adult FA and immune variables studied in insects, only two combinations showed the relationship (Rantala *et al.* 2000; Yourth, Forbes & Smith 2002; Rantala, Ahtiainen & Suhonen 2004). Moreover, one of these two combinations was in crickets (Rantala *et al.* 2004), which have a very gradual metamorphosis throughout the larval stage making it less probable that metamorphosis would erase the relationship between FA and immune variables. With regard to mass, adult mass and FA were also found to be not related in the damselfly *Enallagma ebrium* (Leung & Forbes 1997). Strong links with survival and fitness have been shown for mass at metamorphosis (Sokolovska *et al.* 2000) and PO (Rolff & Siva-Jothy 2004) in adult damselflies. The lack of a relationship between FA after metamorphosis and variables reflecting individual quality (i.e. mass and immunity) may cause the absence of a relationship between FA and fitness. Other mechanisms like random parasitism may also decouple individual quality from adult FA, as has been shown for *C. puella* (Bonn *et al.* 1996). Note, however, this does not exclude a direct link between FA and fitness (independent of individual quality) as individuals with more symmetric wings may have a higher mating success due to flight mechanistic reasons as previously suggested in *C. puella* (Harvey & Walsh 1993) and another damselfly (De Block & Stoks 2007).

In summary, we demonstrated that FA reflected larval stress and individual quality before but not after metamorphosis. The developmental selection hypothesis could not explain this pattern. Instead our data strongly suggest that the often observed lack of relationship between larval stress, adult FA and individual quality (Bjørksten, Fowler & Pomiankowski 2000b; Møller 2006) was due to increased stress levels during metamorphosis, which equalized asymmetry levels among treatments. Our results show that FA may accurately reflect stress and individual quality in individuals with a CLC, however, this will depend on the developmental stage in which the measurements are taken and the developmental stage in which the stress is experienced.

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