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Delayed effects of chlorpyrifos across metamorphosis on dispersal-related traits in a poleward moving damselfly

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Abstract

How exposure to contaminants may interfere with the widespread poleward range expansions under global warming is largely unknown. Pesticide exposure may negatively affect traits shaping the speed of range expansion, including traits related to population growth rate and dispersal-related traits. Moreover, rapid evolution of growth rates during poleward range expansions may come at a cost of a reduced investment in detoxification and repair thereby increasing the vulnerability to contaminants at expanding range fronts. We tested effects of a sublethal concentration of the widespread pesticide chlorpyrifos on traits related to range expansion in replicated edge and core populations of the poleward moving damselfly *Coenagrion scitulum* reared at low and high food levels in a common garden experiment. Food limitation in the larval stage had strong negative effects both in the larval stage and across metamorphosis in the adult stage. Exposure to chlorpyrifos during the larval stage did not affect larval traits but caused delayed effects across metamorphosis by increasing the incidence of wing malformations during metamorphosis and by reducing a key component of the adult immune response. There was some support for an evolutionary trade-off scenario as the faster growing edge larvae suffered a higher mortality during metamorphosis. Instead, there was no clear support for the faster growing edge larvae being more vulnerable to chlorpyrifos. Our data indicate that sublethal delayed effects of pesticide exposure, partly in association with the rapid evolution of faster growth rates, may slow down range expansions.

Capsule: Effects of larval chlorpyrifos exposure impair adult flight-related traits thereby slowing down range expansion.

Key words: carry-over effects, evolutionary ecotoxicology, immunosuppression, organophosphates, rapid evolution

1. Introduction

There is increasing research at the interface of global warming and ecotoxicology, mainly driven by the concern that many contaminants are more toxic at higher temperatures (Moe et al., 2013, Noyes et al., 2009 and Rohr and Palmer, 2013). Besides dealing locally with the temperature increase, populations may also react to global warming by moving polewards and thereby track their temperature envelope (Chen et al., 2011 and Parmesan et al., 1999). Under the latter scenario organisms will not be faced with an exposure to contaminants at higher temperatures. Yet, also under poleward range shifts studying the effect of contaminants is highly relevant for two reasons. Firstly, exposure to contaminants may slow down the range expansion by negatively affecting two types of traits: traits related to the population growth rate (such as individual growth rates, Phillips 2009) and/or traits related to dispersal performance (such as flight ability, e.g, Hill et al., 2011). Secondly, multivariate trait evolution is expected to occur in the edge populations at expanding range fronts (Burton et al., 2010) which may increase the vulnerability to contaminants of edge populations at the moving range front.

A particularly relevant topic in the context of range expansion is whether vulnerability to contaminants in the larval stage can bridge metamorphosis and generate negative delayed effects on adult traits shaping dispersal performance. Many species that are expanding their range have a complex life cycle with larval stages where growth and development occur separated by metamorphosis from adult stages where reproduction and dispersal occur (Stoks and Cordoba-Aguilar, 2012). Ecotoxicological studies are typically carried out in early-life stages which may lead to the underestimation of the effects of contaminants as there is increasing evidence that their negative effects may be carried over from the larval stage to the dispersing adult stage (Distel and Boone, 2009, Janssens et al., 2014, Janssens and Stoks, 2013a, Rohr and Palmer, 2005). Carry-over effects of contaminants on flight-related traits and

on immune function are directly relevant in the context of range expansion as these traits contribute to dispersal ability (Gyulavári et al., 2014; Therry et al., 2014c).

Individual growth rates in the non-dispersive larval stage are predicted to increase in edge populations at the range front because genotypes that are able to produce a larger number of generations in a given amount of time will be favoured by selection at the initial low conspecific densities at the expansion front (Phillips, 2009 and Phillips et al., 2010). The few studies that looked at this, indeed documented the evolution of faster individual growth rates at the edge populations of the moving range front in tadpoles of the cane toad *Bufo marinus* (Phillips, 2009) and in larvae of the damselfly *Coenagrion scitulum* (Therry et al., 2014b). This rapid evolution may increase the vulnerability to stressors at expanding range fronts as an indirect evolutionary outcome (Dmitriew, 2011). Life history predicts animals investing more energy in growth to allocate less energy to detoxification and repair and therefore to be more vulnerable to contaminants (Congdon et al., 2001 and Sibly and Calow, 1989). This generates the prediction that the evolution of faster larval growth rates in edge populations during range expansion is associated with an increased vulnerability to contaminants. This evolutionary mechanism would reinforce the general negative effects of exposure to contaminants on further range expansion in edge populations.

We here studied immediate and delayed effects of a widespread pesticide on traits related to range expansion of a poleward moving insect. We also compared the vulnerability to the pesticide between the core and the edge populations. As study species we chose the poleward range-expanding damselfly *Coenagrion scitulum* which we have been monitoring and studying in detail and where we have shown a rapid evolutionary increase in larval growth rate driven by the range expansion process (Therry et al., 2014b,c). Damselflies are showing larger poleward range shifts under global warming compared to other insect species (Hassall and Thompson, 2010 and Hickling et al., 2006). We exposed *C. scitulum* larvae of

replicated edge and core populations to chlorpyrifos, one of the most used pesticides worldwide (Eaton et al., 2008), in an indoor common garden rearing experiment from the egg stage. In related coenagrionid damselflies, chlorpyrifos has shown to suppress the immune function (Janssens and Stoks, 2013a) and cause carry-over effects to the adult stage (Janssens and Stoks, 2013b).

We tested for sublethal effects of pesticide exposure in the larval stage on four sets of traits relevant for range expansion: larval life history (larval growth and development), metamorphosis success, adult flight-related traits (mass at emergence, flight muscle mass and fat content, Therry et al., 2014c) and adult immune-related traits (phenoloxidase activity and the encapsulation response, Gonzalez-Santoyo and Cordoba-Aguilar, 2012 and Rantala et al., 2011). Any general negative effects of the pesticide on the studied traits are expected to slow down the range expansion, because they reduce the population growth rate (larval growth and development, Phillips, 2009) or the dispersal ability (traits related to flight performance and immune function, Therry et al., 2014c). Furthermore, we tested the hypothesis that edge populations have a higher vulnerability to pesticides because of the evolution of rapid larval growth rates at the range front. This is to be expected because of an energy-mediated trade-off between growth rate, and detoxification and defence (Congdon et al., 2001 and Sibly and Calow, 1989). Because such trade-offs may only become visible at suboptimal food conditions where the total energy available is limited (Reznick et al., 2000 and van Noordwijk and de Jong, 1986) we tested for chlorpyrifos effects at both low and high food levels.

2. Materials and methods

2.1 Study populations and rearing experiment

Coenagrion scitulum (Rambur, 1842) is a Mediterranean damselfly preferring small ponds (Dijkstra, 2006). Up to the 1990s the northern range limit was situated in northern France,

after which a north-eastward range expansion has occurred (Swaegers et al., 2013 and Wasscher and Goudsmits, 2010). In 2010, the northwestern limit of the expanding range margin was situated in the southern parts of The Netherlands, and the northeastern limit in Western Germany (Wasscher and Goudsmits, 2010). Fifteen mated females per population were collected from two core populations (Indre and Nord-Pas-de-Calais) situated within the historical continuous distribution of the species in France, and from two edge populations at the expanding range front (Saarland in Germany, and Zeeland in The Netherlands) that were sampled less than five years after founding (see Appendix S1).

Females were individually placed in plastic cups with wet filter paper as oviposition substrate. Filter papers with eggs were transported to the laboratory in Belgium. Three days after hatching, larvae were individually assigned to plastic vials (7.5 cm height, 3.5 cm diameter) filled with dechlorinated tap water to a height of 6 cm. Larvae were reared 5-7 months in the laboratory before they molted into the final instar. One day after molting into the final instar, larvae were introduced in the pesticide exposure experiment. Throughout the rearing experiment, the temperature was kept at 20°C and a 16:8 L:D photoperiod. Larvae were fed with *Artemia* nauplii *ad libitum* (459 ± 48 nauplii per food portion, mean ± SE, n = 10 food portions counted) five days per week.

2.2 Experimental design

To test whether exposure to the pesticide chlorpyrifos (CPF) affects traits relevant for range expansion and how evolutionary processes during range expansion may further affect the general vulnerability to CPF we set up a full factorial design with 2 population types (core and edge, each represented by two populations) × 2 CPF concentrations (present and absent) × 2 food levels (low and high). Larvae were individually exposed to the treatments throughout the entire final instar. In the low food treatment larvae were fed *Artemia* nauplii three times

per week. In the high food treatment, larvae were fed *Artemia* nauplii six times per week supplemented with three *Chironomus* larvae per week. The number of *Artemia* nauplii given per feeding session was the same as during the pre-exposure period (see Appendix S1). The number of larvae exposed per combination of population type × CPF concentration × food level ranged between 66 and 83 (total of 597 larvae). Exact sample sizes per response variable are presented in the figures.

Larvae in the treatment with CPF received a pulse application every three days throughout the final instar with renewal of the medium. We used a CPF concentration of 0.57 µg/l (measured directly after renewal based on a pooled sample of 10 vials). The CPF concentration was analyzed by the research laboratory Lovap NV (Geel, Belgium) using gas chromatography in combination with mass spectrometry. This CPF concentration was chosen as we observed mortality in another coenagrionid damselfly at higher CPF concentrations (Dinh Van et al., 2014), while in the current study we were especially interested in delayed sublethal effects of CPF on the dispersal-related traits (flight-related traits and immune function). This CPF concentration is realistic in water bodies adjacent to agricultural lands (e.g., Bernabo et al., 2011 and Zhang et al. 2012) where the study species can be abundant. A stock solution obtained by dissolving CPF powder (Sigma-Aldrich, purity > 99%) in absolute ethanol was kept in amber glass bottles and stored in the dark at 4°C. The CPF exposure solution was prepared three times per week by diluting the stock solution with synthetic pond water (for details see Janssens and Stoks, 2013c). After 72h (just before renewal, hence the expected lowest exposure concentration) the CPF concentration had decreased to 0.03 µg/l. Synthetic pond water was used as a control; growth rates of damselfly larvae in this synthetic pond water do not differ from those observed in natural pond water (Janssens and Stoks, 2013c). Note that we did not use ethanol as a solvent control since a previous experiment has shown no detectable effects of 5 µl/l ethanol on growth of damselfly larvae (L. Janssens,

unpublished data), while the ethanol concentration to which larvae were exposed in this study was 1.5 µl/l which is also ca. 6,000 times lower than the lowest reported NOEC for aquatic invertebrates (United Nations Environment Program, 2004).

2.3 Response variables

We daily checked the mortality during the exposure period. Larval survival was calculated as the percentage of larvae at the start of the exposure period that survived up to metamorphosis. Larval growth rate was calculated as the mass increase during the first 15 days of the exposure period; a preliminary test showed that the first adults emerged at day 16 of the exposure period. Larvae were weighed before (initial larval wet mass) and at day 15 (final larval wet mass) to the nearest 0.01 mg using an electronic balance (Mettler Toledo® AB135-S). Individual larval growth rates were calculated as $(\ln_{\text{final mass}} - \ln_{\text{initial mass}})/15$ days (see Stoks et al., 2012). Development time was calculated as the duration of the final instar.

We daily checked for emergence and coded the metamorphosis success using three ordered categories: 0 (= animals that did not fully cast their larval skin and died during metamorphosis), 1 (= animals that fully casted their larval skin but had at least one not fully extended wing), and 2 (= animals that fully casted their larval skin with four normally extended wings). After emergence, adults were kept in the dark for 16 hours to allow hardening of the exoskeleton. All adults were then weighed to the nearest 0.01 mg using an electronic balance (Mettler Toledo® AB135-S).

We immune-challenged half of the adults that successfully emerged per population (n = 328) by inserting a nylon monofilament and subsequently measured the degree of encapsulation (see Appendix S2 for details). This is a standard method to measure the immune response in insects and mimics an ectoparasitic water mite attack in damselflies (Rantala et al., 2000, 2010 and Rolff and Siva-Jothy, 2004). Damselflies defend themselves

against ectoparasitic water mites by encapsulating the feeding tubes of the externally located mites (Rantala et al., 2010) in a similar way as they encapsulate the implant. The encapsulation response was expressed as the mean of the grey values from three digitalized photos at three different angles of the nylon monofilament.

To prepare animals for physiological assays, thorax and abdomen were homogenized separately in cacodylate buffer (0.01 mol/l Na-Coc, 0.005 mol/l $CaCl_2$) with a dilution factor of 15. Thereafter, samples were centrifuged for 10 minutes (4ºC, 13,200 g) to remove the cell walls and the exoskeleton. Subsequently, supernatants of thorax and abdomen were pipetted to separate eppendorf tubes. Twenty microliter of the thorax supernatant was used for quantifying the thorax protein content, a proxy for flight muscle mass (Snell-Rood et al., 2014). This was done using the Bradford method (Bradford, 1976). Relative flight muscle mass was expressed as µg protein in the thorax / mg thoracic wet mass.

The remaining of the thorax supernatant was mixed with the abdomen supernatant to quantify phenoloxidase (PO) activity and fat content. We assayed the activity of PO, a key component of the insect immune system (Gonzalez-Santoyo and Cordoba-Aguilar, 2012), using the protocol of Stoks et al. (2006). PO activity was measured as the slope during the linear phase of the reaction curve (time lapse 1000-2000s) when the enzyme catalyses the transition from L-DOPA to dopachrome and was expressed as µmol dopachrome formed per min. Total protein content was used as a covariate. Total fat content, an important energy reserve in insects (Arrese and Soulages, 2010), was quantified based on the method of Bligh and Dyer (1959). Relative fat content was expressed as µg fat/mg adult wet mass. All physiological variables were measured in duplicate and the mean of both readings per sample was used for statistical analyses.

2.4 Statistical analyses

To test for effects of population type (core vs edge), the CPF treatment and food level and all their interactions on survival (binary, 0 vs 1) during the larval exposure period we used a linear mixed model with a binomial error structure and the logit link function (Bolker et al. 2009, Warton and Hui, 2011) using the glimmix procedure in SAS v9.3 (SAS Institute Inc., Cary, NC, USA). Model validation showed a good fit (dispersion parameter = 1.04) (Crawley, 2002). The analysis of metamorphosis success (ordered categories: 0, 1 or 2) was performed using a generalized linear mixed model with an ordered multinomial error structure and the cumlogit link (Bolker et al. 2009). This error structure takes into account the ordered nature of the multinomial response. Also for this variable, model validation showed a good fit (dispersion parameter = 1.02) (Crawley, 2002).

All other response variables (larval growth rate, development time, mass at emergence, encapsulation response, PO activity, fat content, and flight muscle mass) were normally distributed and therefore analyzed using linear mixed models (LMMs) with a normal error structure and the identity link (Bolker et al. 2009). These models tested for effects of the fixed effects population type, CPF treatment and food level and all their interactions using the mixed procedure of SAS v9.3. For traits measured after the implant treatment (PO activity, fat content and relative flight muscle mass), the implant treatment (and its interactions) was also included as a fixed effect in the analyses (Dinh et al., 2016).

In all models, population nested in population type was initially included as a random factor (following e.g. Wash and Post, 2011). Whenever this random factor population had a p-value > 0.10, we simplified the final model by removing this term (Crawley 2002). Note that when the random nested factor population remained in the model this affected the degrees of freedom for the fixed effect population type as the denominator to calculate the F-statistic is

then changed (from the MS of the error term into the MS of the random term population nested in type) (Neter et al., 1996).

For all LMMs, we tested the assumptions of normality of the error distributions with Shapiro-Wilk tests and the homogeneity of variances with Levene's tests (Quinn and Michael, 2002). Final instar duration, relative flight muscle, relative fat content, and PO activity were log(x + 1)-transformed to meet model assumptions. Adult mass was included as a covariate when analyzing the encapsulation response and PO activity (Therry et al., 2014c). The total protein concentration was included as a second covariate when analysing PO activity (Therry et al., 2014c).

In damselflies, sexes may differ in their response to stressors such as pesticide exposure (e.g., Campero et al., 2008a) and food shortage (e.g., Mikolajewski et al., 2007). We therefore sexed all adults at emergence. Note that although larvae were reared and exposed individually, the large number of larvae (ca. 600) made it logistically not possible to sex all larvae at the start of the exposure period. Also we could not trace back the sex for all larvae given mortality during metamorphosis (see results). We could therefore only separately analyse per sex the response variables measured in successfully metamorphosed adults (mass at emergence, relative flight muscle mass, fat content, encapsulation response and PO activity).

For clarity, we report the results of the full statistical analyses in supplementary tables (Appendix S3) and include only relevant statistical information in the results section. When there was a main effect of population type or an interaction between population type and a stressor, we provide additional figures showing the variability between the two edge populations and between the two core populations (Appendix S4). To keep the manuscript focused and to avoid too complex figures we only present figures on the effects of CPF and

their significant interactions for those response variables where also the nylon implant was added to the statistical models. Full-factorial figures for these variables and a full description of all effects, including implant effects, are presented in Appendix S5.

3. Results

3.1 Larval life history traits

Larval survival did not depend on the presence of CPF and did not differ between core and edge populations (Fig. 1A-B, Table S1 in Appendix S3). Larvae reared at low food had lower survival than larvae reared at high food ($F_{1, 587} = 25.81$, $P < 0.001$); this pattern was similar across population types and CPF levels (Fig. 1A-B, Table S1).

Larval growth rate was not affected by CPF, nor did CPF interact with population type or food level (Fig. 1C-D, Table S1). Larvae reared at low food grew slower than larvae reared at high food ($F_{1, 578} = 1878.34$, $P < 0.001$, Fig. 1C-D). Edge larvae had higher growth rates than core larvae but only when reared at high food while their growth rate did not differ at low food (Population type × Food interaction, $F_{1, 578} = 5.71$, $P = 0.017$, Fig. 1C-D). This pattern was consistent between the two edge and the two core populations (Fig. S1 in Appendix S4).

CPF did not affect the duration of the larval period (Fig. 1E-F, Table S1). Development times of larvae from edge and core populations did not differ (Fig. 1E-F). Larvae reared at low food had a longer developmental time than those reared at high food ($F_{1, 530} = 444.56$, $P < 0.001$, Fig. 1E-F). There were no interactions between population type, food level and the CPF treatment (Table S1).

3.2 Metamorphosis success

In general, edge animals had a lower metamorphosis success than core animals ($F_{1, 529} = 4.59$, $P = 0.032$, Fig. 2 and S2, Table S2 in Appendix S3). Exposure to CPF reduced

metamorphosis success ($F_{1, 529}$= 7.14, P = 0.0078); this was mainly because exposure to CPF increased the proportion of adults with malformed wings by a factor of three (4% at 0 µg/l CPF vs. 13% at 0.57 µg/l CPF, Fig. 2). There was a trend for a Type × CPF interaction ($F_{1, 529}$ = 3.23, P = 0.073, Fig. 2) indicating a stronger CPF effect on metamorphosis success in core populations; this pattern was consistent across the two edge and the two core populations (Fig. S2 in Appendix S4). Animals reared at low food had a lower metamorphosis success than animals reared at high food ($F_{1, 529}$ = 24.36, P < 0.001). The effects of food and population type were mainly driven by mortality during metamorphosis. When CPF exposure was combined with low food, their additive combination resulted in the lowest percentage (54% compared to 84% in the control without stressors) of animals that emerged with normal wings.

3.3 Adult flight-related traits

Population type did not affect mass at emergence (Fig. 3, Table S2). While CPF did not affect male mass, female mass was lower in the presence of CPF (Females: $F_{1, 225}$ = 5.93, P = 0.016; Fig 3C-D). In both sexes, mass at emergence was lower in adults reared as larvae at low food than at high food (Males: $F_{1, 192}$ = 118.79, P < 0.001; Females: $F_{1, 225}$ = 190.95, P < 0.001, Fig. 3). As a result of additive effects, female mass at emergence was 30% lower compared to the control without stressors when CPF was combined with low food (Fig. 3C).

There were no overall main effects of CPF exposure and population type on relative flight muscle mass (Figure 4A-B, Table S3). In males, there was a significant Population type × CPF interaction ($F_{1, 170}$ = 5.63, P = 0.018): exposure to CPF reduced the relative flight muscle mass in edge populations but not in core populations; this pattern was independent of food level (Population type × Food × CPF, $F_{1, 170}$ = 1.69, P = 0.20, Fig. 4A), and consistent across the two edge and the two core populations (Fig. S3). In females, the flight muscle mass was not consistent across the two edge and the two core population (Population nested in

population type, $F_{2, 199} = 3.51$, $P = 0.032$), with flight muscle mass being lowest in core population Indre and edge population Saarland, higher in edge population Zeeland and highest in core population Nord-Pas-de-Calais (Fig. S4 in Appendix S4). At low food and in the absence of CPF, edge females had a lower relative flight muscle mass than core females (Population type × Food × CPF interaction, $F_{1, 213} = 4.76$, $P = 0.030$, Fig. 4B). This pattern was consistent across the two edge and the two core populations (Fig. S5 in Appendix S4). In both sexes, the relative flight muscle mass was lower in adults reared as larvae at low food (Males: $F_{1, 170} = 16.60$, $P < 0.001$; Females: $F_{1, 214} = 92.98$, $P < 0.001$, Fig. 4A-B). As a result of additive stress effects, flight muscle mass of edge males was reduced with 10% when CPF was combined with low food (Fig. 4A).

Exposure to CPF tended to reduce the relative fat content in females ($F_{1, 200} = 3.04$, $P = 0.083$, Fig. 4D, Table S3). In males, there was a trend for a CPF × Type interaction ($F_{1, 185} = 3.78$, $P = 0.053$) but there was no consistent CPF effect across the two edge and the two core populations (Fig. S6A): one core population (Indre) and one edge population (Zeeland) tended to reduce fat content in the presence of CPF, while the reserve trend was observed in the other core population (Nord-Pas-de-Calais) and the other edge population (Saarland). In both sexes, the relative fat content was lower in adults reared as larvae at low food (Males: $F_{1, 179} = 25.34$, $P < 0.001$; Females: $F_{1, 200} = 40.45$, $P < 0.001$, Fig. 4C-D). Relative fat content tended to be lower in core females than in edge females ($F_{1, 200} = 3.25$, $P = 0.073$), and in core males than in edge males when reared at high food (Population type × Food, $F_{1, 169} = 3.81$, $P = 0.052$, Fig. 4C-D). These patterns were consistent across the two edge and the two core populations (Fig. S6B and S6C in Appendix S4). As a result of additive stress effects, relative fat content was 36% lower in females exposed to CPF and low food (Fig. 4D).

3.4 Adult immune function

Larval exposure to CPF reduced the encapsulation response in adult males ($F_{1, 94} = 4.63$, $P = 0.034$) but not in adult females (Fig. 5A-B). In neither sex there was a difference in the encapsulation response between core and edge populations or between food levels (Fig. S10A-B in Appendix S5). There were no interactions between population type, food level and CPF on the encapsulation response (Table S4).

On average, edge males had a higher PO activity than core males ($F_{1, 168} = 4.47$, $P = 0.036$, Fig. 5C); this differentiation between core and edge populations was not present in females (Fig. S8). Exposure to CPF reduced the PO activity only in males of edge populations when they were subsequently challenged with an implant (Population type × CPF × Implant interaction, $F_{1, 168} = 4.17$, $P = 0.043$, Fig. 5C). Because edge males with an implant had a higher PO activity than core males with an implant in the absence of CPF, this reduction resulted in equal PO activities in core and edge males in the presence of CPF. These effects were consistent across the two edge and the two core populations (Fig. S7). CPF had no effect on the PO activity of females ($F_{1, 215} = 0.01$, $P = 0.93$, Fig. S10 D-F). Food level had no overall effect on the PO activity of males (Table S4) but high food increased the PO activity stronger in edge females than in core females ($F_{1, 215} = 3.92$, $P = 0.049$); this pattern was consistent across the two edge and the two core populations (Fig. S8 in Appendix S4).

4. Discussion

The imposed low food level had considerable negative effects on nearly each trait measured. Instead, the effects of the pesticide, as expected for a concentration that was sublethal during the exposure period, were more subtle and only detectable at and after metamorphosis. Edge and core populations showed consistent differences across treatments for several traits including a higher growth rate in edge populations (at high food), yet there was no clear support this resulted in a higher vulnerability to CPF exposure. To structure the discussion we will first discuss the general effects of low food and exposure to the pesticide, and then focus

on the differentiation between core and edge populations including differential responses of both population types to the stressors.

4.1 Food effect

Overall, food availability had strong effects on life history and flight-related traits, yet had almost no effect on the immune response of *C. scitulum*. Food shortage generally results in suboptimal values of fitness-related traits in insects (Nylin and Gotthard, 1998) including damselflies (Stoks and Cordoba-Aguilar, 2012) due to energetic constraints. In line with this, *C. scitulum* larvae reared at low food had a considerable lower survival (both during the larval period and during metamorphosis) and growth rate, a longer larval period, and in the adult stage a smaller mass at emergence, a lower flight muscle mass and a lower fat content. These effects of the low food level likely negatively affect fitness. For example, the extended larval period, may increase the probability of being exposed to multiple pulses of pesticides in ponds in agricultural areas where pesticides are sprayed frequently (Van Drooge et al., 2001). The smaller mass at emergence, the lower flight muscle mass and fat content may reduce the dispersal ability (e.g. Anholt, 1990) and mating success (e.g. Swillen et al., 2009).

4.2 Pesticide effect

A key finding in our study is that exposure to a sublethal CPF concentration had no detectable immediate negative effects throughout the exposure period that spanned the entire final instar (ca. 23 days at high food and ca. 36 days at low food), yet reduced traits related to range expansion during and after metamorphosis. Indeed, we did not find any effect of CPF on larval survival, growth rate and development time. Yet, exposure to CPF in the larval stage negatively affected flight-related traits and immune traits in the adult stage. These carry-over effects of CPF exposure across metamorphosis likely reduce fitness through their effect on flight ability (Stoks and Cordoba-Aguilar, 2012) and parasite resistance (Yourth et al., 2002).

Specifically, exposure to 0.57 µg/l CPF negatively affected several flight-related traits: it resulted in a 3× higher percentage of wing malformation, a lower mass at emergence (females), a lower relative flight muscle mass (edge males) and a trend for a lower fat content (females). Previous studies have shown that exposure to organophosphate or organochlorine pesticides can result in increased malformation of locomotor structures. For example, exposure to chlorpyrifos at much higher concentrations of 25-100 µg/l resulted in tail flexure and skeletal and muscle defects in tadpoles (Bernabo et al., 2011), and exposure to endosulfan at concentrations of 0.03-5 µg/l caused a failure to develop fore- and hind-limbs in frogs (Devi and Gupta, 2013). These malformations have been explained by the pesticide-induced alteration in body wall muscles. Possibly, a CPF-induced alteration in the muscles also occurred in the damselfly larvae and thereby lowered their ability to cast their larval skin. This is suggested by the fact that CPF-exposed larvae also needed more time to complete metamorphosis (K. V. Dinh, personal observation). The CPF-induced reductions in body mass, relative flight muscle mass and fat content likely were due to a lower food intake (Dinh Van et al., 2014) combined with a higher investment in detoxification and repair in the larval stage as has been documented before in another damselfly species (Janssens and Stoks, 2013b).

Besides the flight-related traits, exposure to the here used sublethal concentration of CPF in the larval stage also had an immunosuppressive effect in adult males as measured by reductions in the encapsulation response and in PO activity (in edge males that received an implant). Such negative effects of organophosphate pesticides on immune function have been well-documented (reviewed in James and Xu, 2012), yet mainly within the same life stage in which animals were exposed to the pesticide (but see e.g. Campero et al., 2008a; Janssens et al. 2014). For example, Janssens et al. (2014) showed that larval exposure to 2 µg/l CPF reduced PO activity in adults of the damselfly *Ischnura elegans.* A reduction in PO activity

was also found in the damselfly *C. puella* exposed to 30 µg/l of endosulfan (Campero et al., 2008a). Such immunosuppressive carry-over effects across metamorphosis may make animals that were exposed to pesticides in their larval stage more vulnerable to pathogens and parasites in the adult stage (e.g. Rohr et al., 2013). The reduction in the encapsulation response observed in the present study has been associated with a lower probability of damselflies to kill engorging ectoparasitic water mites (Yourth et al., 2002). That the immunosuppression was only seen in males may reflect the idea that immune function is more important in females than in males (Rolff, 2002). This is because in females, including *Coenagrion* species (Thompson et al., 2011), reproductive success is much more dependent on lifespan than in males, hence females should invest more in defense mechanisms guaranteeing a long adult lifespan. Note that linked to this, sexes may have differently traded off mass and immune function (Rantala and Kortet, 2003) with only the females reducing mass under CPF exposure.

4.3 Differentiation between edge and core populations

Edge populations at an expanding range front undergo selection for a more rapid life history because of r-selection associated with the initial lower population densities at the expansion front (Burton et al., 2010, Phillips, 2009 and Phillips et al., 2010). In line with this, we observed a higher larval growth rate in the two edge populations at the moving range front as compared to the two studied core populations. This confirms the pattern of faster growth in previous studies on sets of four edge (including two edge populations used in the current study) and two core populations of the study species in a similar common-garden study (Therry et al., 2014b) suggesting this pattern to be general. Similarly, larval cane toads grew faster in the edge populations at the moving range front (Phillips, 2009). This faster growth in edge populations was in the current study only seen at high food, suggesting that edge larvae could not maintain their fast larval growth rate at low food due to energetic constraints.

Congruent with selection for a higher dispersal ability at moving range edges (Hill et al., 2011), edge animals (males at high food, and a general trend in females) also tended to have a higher fat content. In the related *C. puella* it was indeed shown that a higher fat content covaried positively with flight endurance (Gyulavári et al., 2014). Given that animals were reared from the egg stage in a common garden experiment, the faster growth rate and higher fat content in the edge populations likely reflect genetic differences, although we cannot fully exclude a contribution of maternal effects (but see Shama et al., 2011 and Strobbe and Stoks, 2004).

While edge individuals invested more in rapid larval growth and adult fat content this was at the expense of a higher mortality during metamorphosis in the absence of CPF. Costs of rapid larval growth have been well-studied (Dmitriew, 2011), yet an increased mortality during metamorphosis has not been documented so far. This may, however, not be that surprising as metamorphosis is an energetically demanding process (Fischer et al., 2005), and rapid larval growth is associated with higher levels of stress as reflected in an increase in asymmetry (Campero et al., 2008b) and an increase in oxidative stress (Gaupale et al., 2012).

Edge males had an overall higher PO activity than core males. This confirms a previous study of *C. scitulum* (Therry et al., 2014c) and may be explained by the range expansion process per se. Indeed, an increased immune function may be a by-product of selection for increased dispersal rates since the ability to disperse can be reduced in animals with a higher pathogen load (Nagel et al., 2010). The higher PO activity in edge males did not translate into a higher encapsulation response, although this has been observed before in this species (Therry et al., 2014c). Possibly, the longer insertion time used by Therry et al. (2014c) (12 h versus 7 h in our study) may cause this difference. The insertion of an implant strongly reduced the PO activity and can be explained by the rapid utilisation of PO in the immune response and a subsequent failure to replenish the supply (Laughton et al., 2011).

The general belief is that faster growing animals should be more vulnerable to contaminants such as pesticides because of a resource-based allocation trade-off whereby increased investment in growth comes at the expense of reduced investment in detoxification and repair (Congdon et al., 2001 and Sibly and Calow, 1989). In line with this trade-off scenario, *Ischnura elegans* damselfly larvae of faster growing southern populations suffered more when exposed to CPF in terms of growth rate (Dinh Van et al., 2014). Here, we found no clear support for this trade-off. Instead, we detected a trend that the negative effect of CPF in increasing wing malformations was stronger in core populations. This may be linked to the observation that the baseline level (without CPF) of wing malformations was already higher in edge populations. The only limited support for a higher vulnerability to CPF exposure in edge populations came from the observation that CPF negatively affected flight muscle mass and PO activity (when implanted) in the faster growing edge males and not in the core males. This resulted in edge males exposed to CPF having the lowest flight muscle mass; the PO activity of edge and core males became, however, equal when exposed to CPF and implanted.

4.4 Conclusions

An increasing number of studies are reporting carry-over effects of larval exposure to pesticides on adult traits (e.g., Alexander et al., 2008, Bridges, 2000, Janssens and Stoks, 2013b and Rohr and Palmer, 2005). These studies typically also documented immediate negative effects during the larval stage. Our study, however, differs in this important aspect and the apparent lack of effects in the larval stage urges caution when running ecotoxicological tests that are limited to only one life stage.

We documented multivariate phenotypic differentiation in ecologically relevant traits between edge and core animals, both in the larval and in the adult stage reflecting the rapid evolutionary processes associated with the range expansion under global climate change and the associated trade-offs. Yet, while edge populations showed the predicted higher growth

rates which were potentially traded off against a lower metamorphosis success, there was no clear support for the general belief that faster growing animals suffer more from contaminants (Congdon et al., 2001 and Sibly and Calow, 1989). This indicates that this evolutionary trade-off scenario, while it may contribute to slowing down range expansions, may be too simplistic as a predictive framework to fully capture the impact of contaminants during range expansion. Irrespective of the life history evolution driving the vulnerability to contaminants, our data indicate that pesticides such as CPF, whose levels are predicted to increase under global climate change (Kattwinkel et al., 2011), have the potential to negatively affect dispersal-related traits at sublethal concentrations (see also in honeybees, Blanken et al., 2015) and therefore may slow down the speed of further expansion at the range front.

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Figure legends

Figure 1. Percentage survival (A, B), mean (+ 1 SE) growth rate (C, D) and development time (E, F) of final instar *Coenagrion scitulum* larvae as a function of chlorpyrifos concentration, food level and population type. Numbers above the bars represent sample sizes.

Figure 2. Metamorphosis success (%) of the damselfly *Coenagrion scitulum* as a function of chlorpyrifos concentration, food level and population type.

Figure 3. Mean (+ 1 SE) mass at emergence of *Coenagrion scitulum* as a function of sex, chlorpyrifos concentration, food level and population type. Numbers above the bars represent sample sizes.

Figure 4. Mean (+ 1 SE) flight muscle mass (A-B), and relative fat content (C-D) of adult *Coenagrion scitulum* as a function of sex, chlorpyrifos concentration, food level, population type. Numbers above the bars represent sample sizes.

Figure 5. Mean (+ 1 SE) encapsulation response (A, B) and phenoloxidase activity (C) of adult male *Coenagrion scitulum* as a function of sex, chlorpyrifos concentration, population type, insertion of the nylon monofilament. Numbers above the bars represent sample sizes. Given are least-square means (with 1 SE) corrected for mass and total protein content.

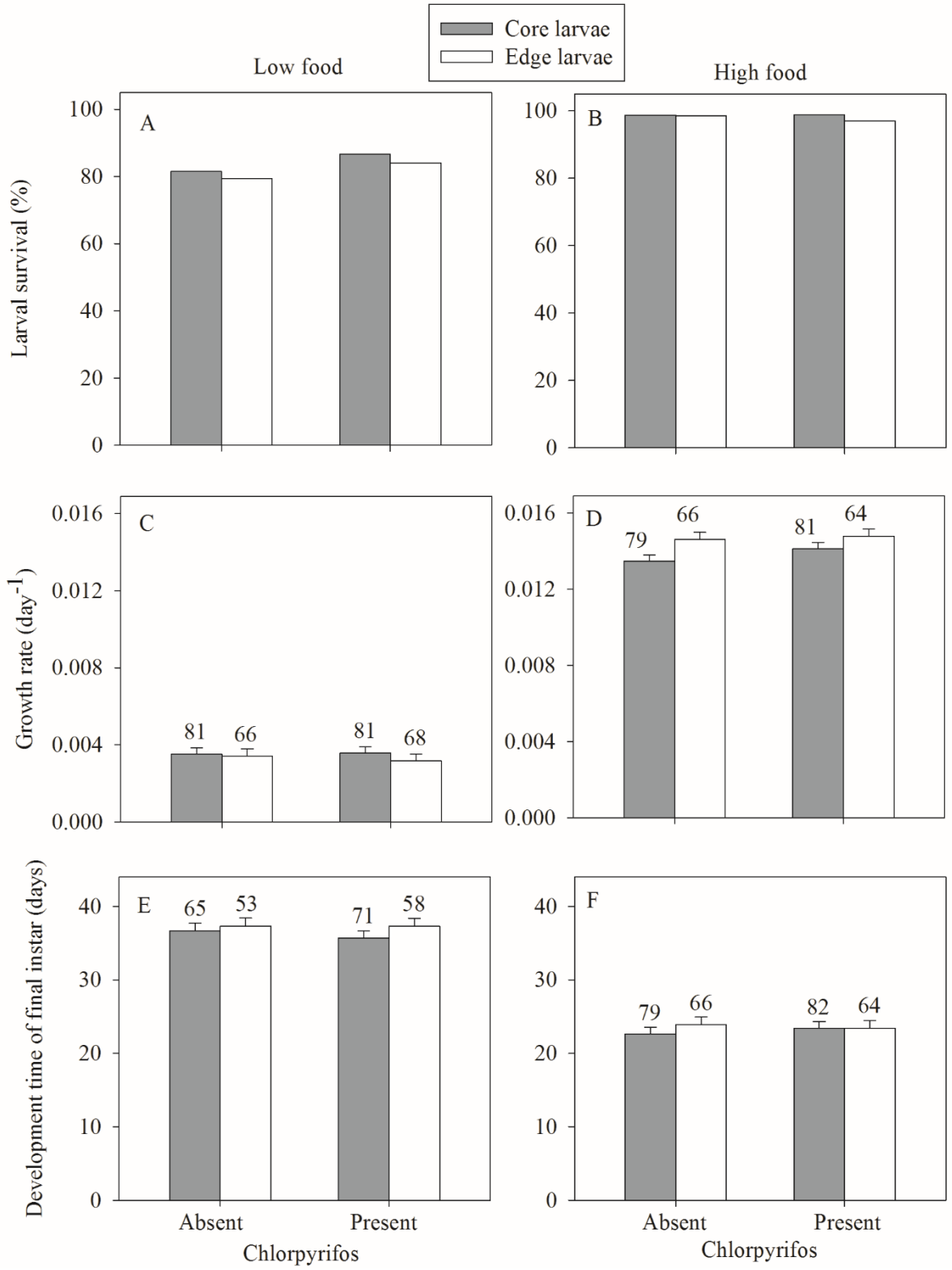
Core larvae
Edge larvae
Low food
High food
A
B
C
D
E
F
Larval survival (%)
Growth rate (day-1)
Development time of final instar (days)
81
66
81
68
79
66
81
64
65
53
71
58
79
66
82
64
Absent
Present
Chlorpyrifos

Figure 1

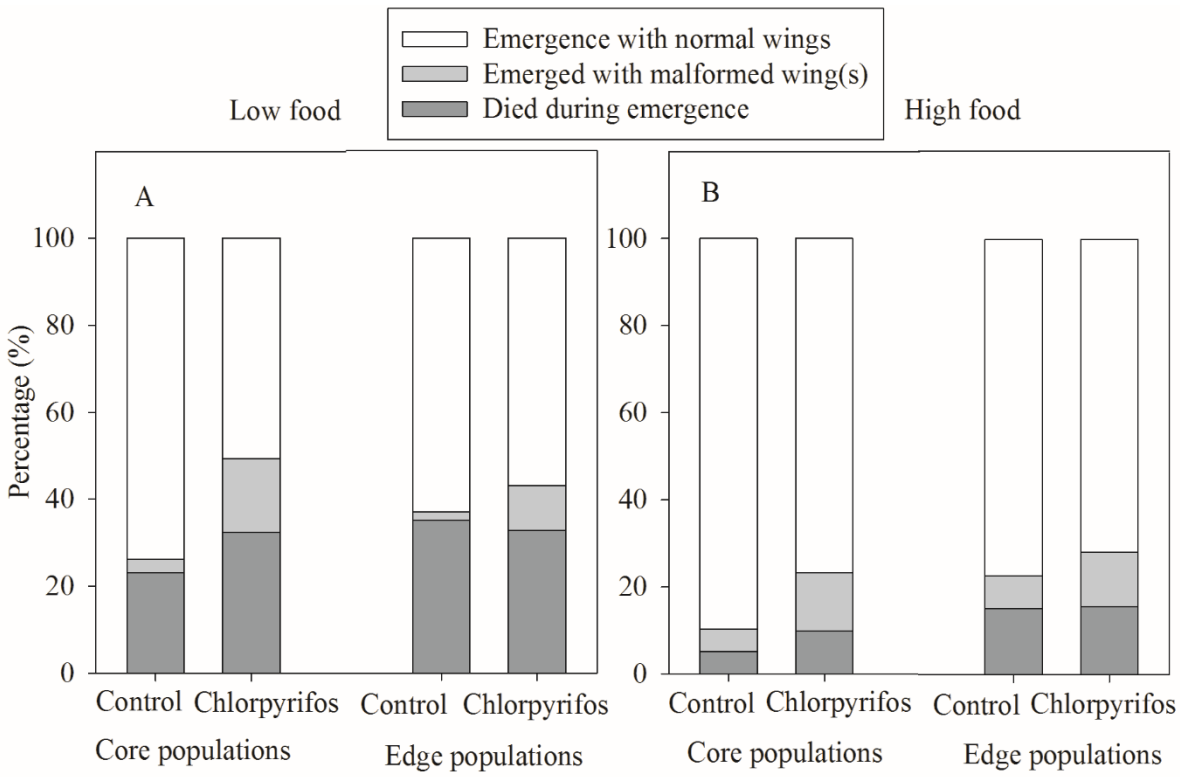
Emergence with normal wings
Emerged with malformed wing(s)
Died during emergence
Low food
High food
A
B
Percentage (%)
100
80
60
40
20
0
Control
Chlorpyrifos
Control
Chlorpyrifos
Core populations
Edge populations
Control
Chlorpyrifos
Control
Chlorpyrifos
Core populations
Edge populations

Figure 2

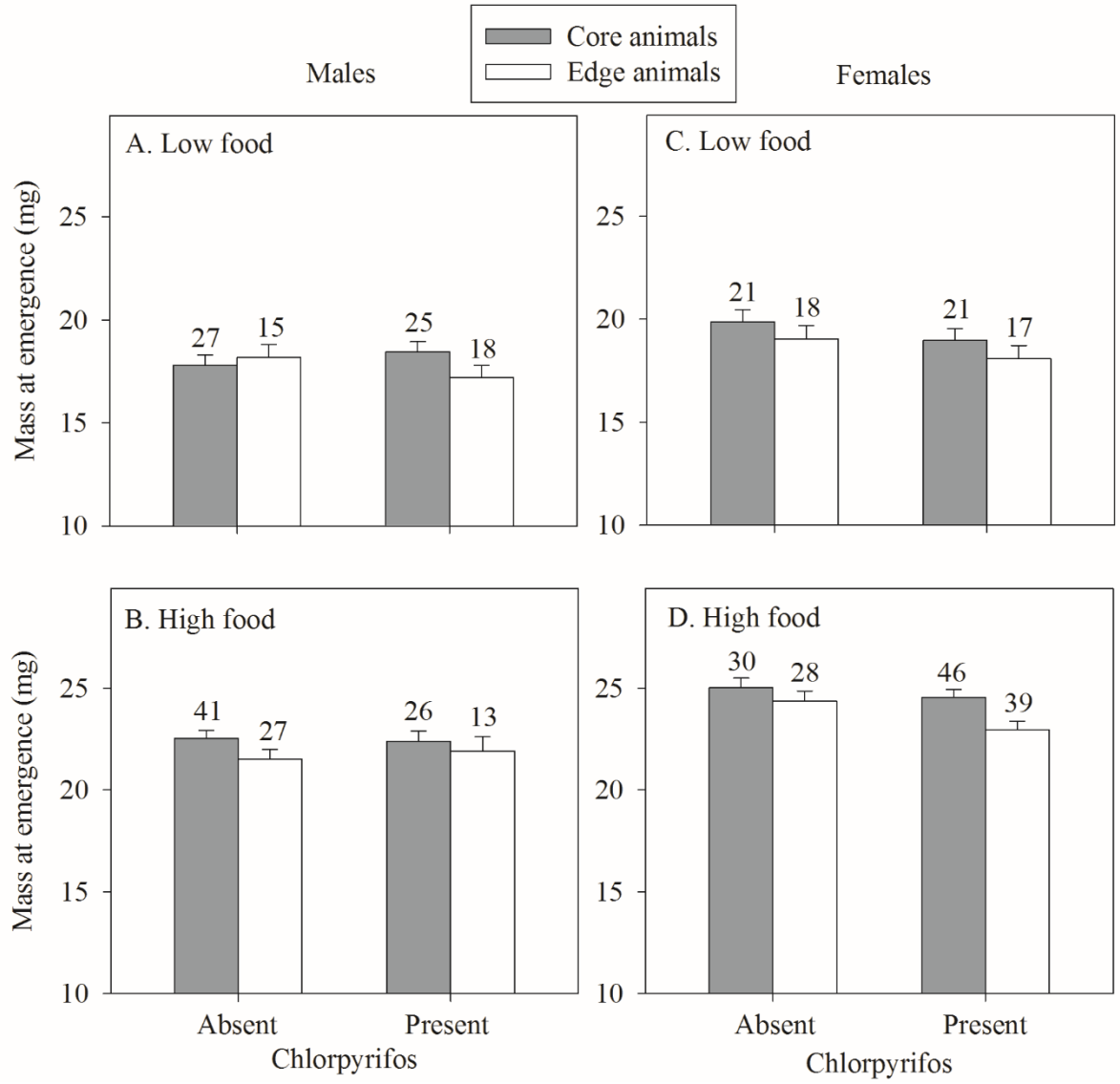
Core animals
Edge animals
Males
Females
A. Low food
C. Low food
B. High food
D. High food
Mass at emergence (mg)
25
20
15
10
27
15
25
18
21
18
21
17
41
27
26
13
30
28
46
39
Absent
Present
Chlorpyrifos

Figure 3

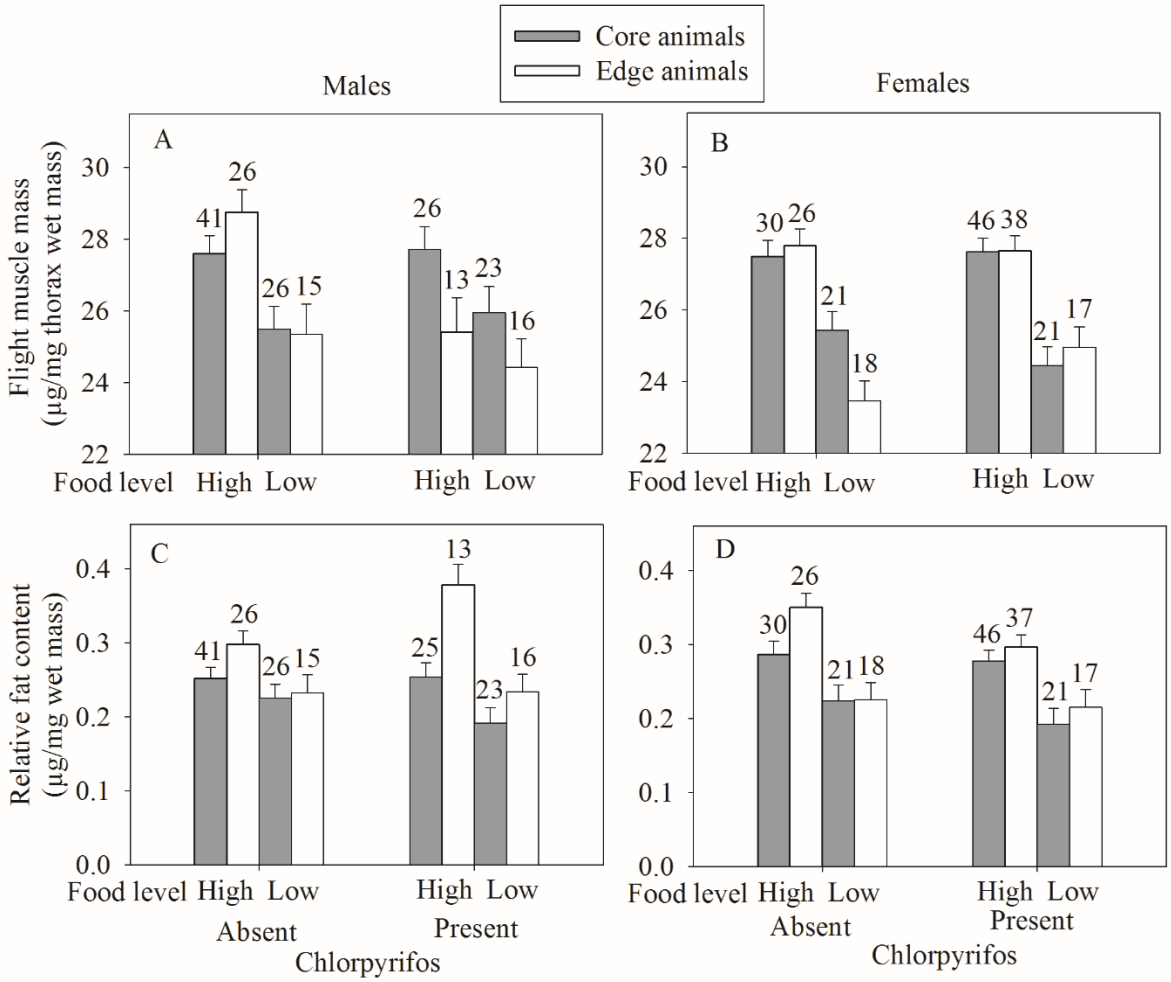
Core animals
Edge animals
Males
Females
A
B
C
D
Flight muscle mass
(µg/mg thorax wet mass)
Relative fat content
(µg/mg wet mass)
Food level
High
Low
Absent
Present
Chlorpyrifos

Figure 4

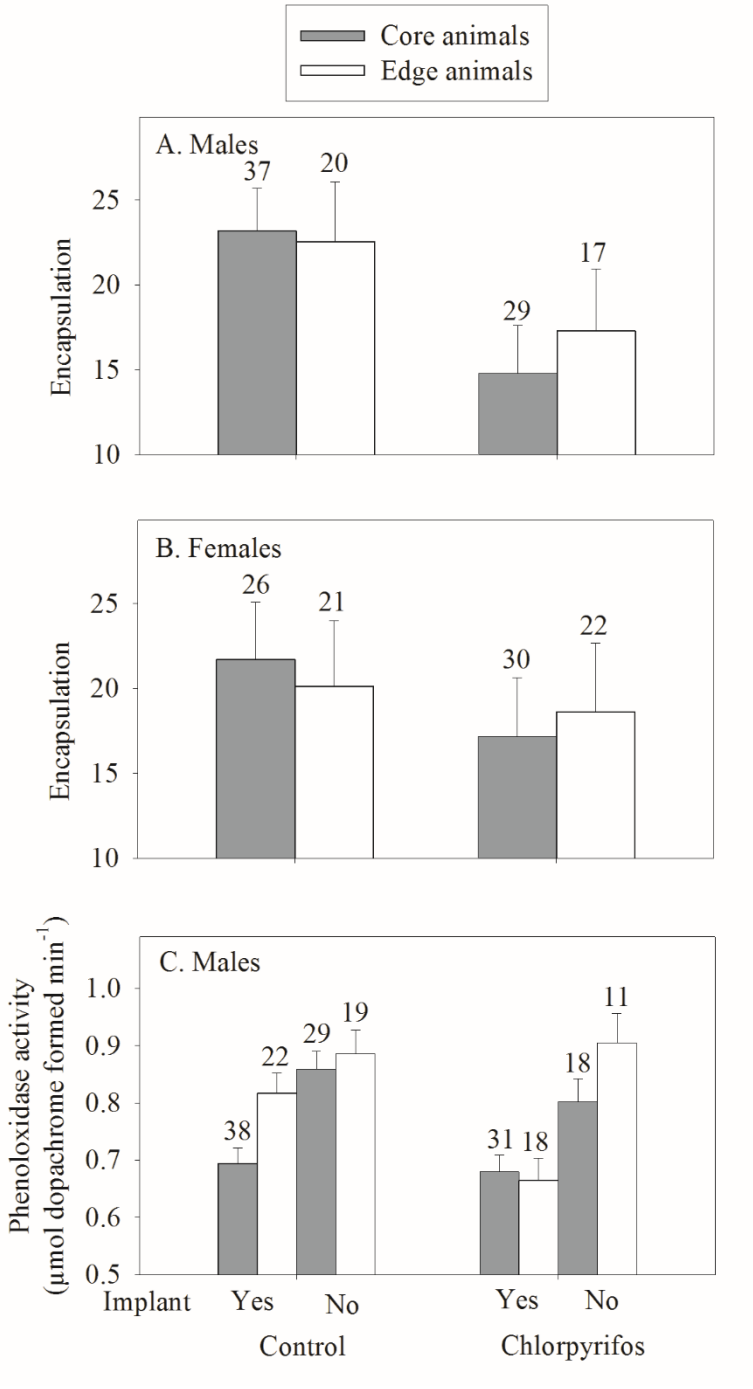
Core animals
Edge animals
A. Males
Encapsulation
25
20
15
10
37
20
29
17
B. Females
Encapsulation
25
20
15
10
26
21
30
22
C. Males
Phenoloxidase activity
(µmol dopachrome formed min-1)
1.0
0.9
0.8
0.7
0.6
0.5
38
22
29
19
31
18
18
11
Implant
Yes
No
Yes
No
Control
Chlorpyrifos

Figure 5

Appendix S1. Study populations and general rearing procedure

The two studied core populations were within the historical distribution of the species in France (Therry et al., 2014). As core populations we selected Indre (46°43’14.03”N, 1°10’20.22”E) and Nord-Pas-de-Calais (50°26’34.37”N, 1°35’08.81”E). As edge populations we selected populations at the moving range front in Germany (Saarland, + 49°14’52.96”N, + 7°16’20.08”E) and in The Netherlands (Zeeland, 51°21’25.99”N, 3°40’01.37”E). Note that as we were interested in the signal of the range expansion process, stable edge populations were not considered as edge populations. The distances between populations varied between 180 and 420 km. All populations are genetically differentiated from each other based on neutral genetic markers (Swaegers et al., 2013; 2015).

The two core populations at Indre and Nord-Pas-de-Calais, and the edge population in Zeeland are in natural areas without agriculture and therefore unlikely to be affected by pesticides (Coors et al., 2009 and Cothran et al., 2013). The edge population in Saarland is within an agricultural area. Any local adaptation to pesticide exposure in this edge population, however, would make any support for our hypothesis of a higher vulnerability to pesticides in edge populations at moving range fronts conservative.

Mated females (15 per population) were collected in June and July 2011 and allowed to oviposit in glass vials with wet filter paper *in situ*. Eggs were transported to the laboratory in Belgium. Three days after hatching, larvae were individually assigned to plastic vials (7.5 cm height, 3.5 cm diameter) filled with dechlorinated tap water to a height of 6 cm. Larvae molted into the final instar after 5-7 months. One day after molting into the final instar, larvae were introduced in the pesticide exposure experiment. Throughout the experiment, the temperature was kept at 20°C and a 16:8 L:D photoperiod. Larvae were fed *Artemia* nauplii

ad libitum (459 ± 48 nauplii per food portion, mean ± SE, n = 10 food portions counted) five days per week.

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Appendix S2

Encapsulation assay

We immune-challenged a subset of 328 adults (half of the adults that successfully emerged per population) by inserting a nylon monofilament and subsequently measured the degree of encapsulation. This is a standard method to measure the immune response in insects and mimics an ectoparasitic water mite attack in damselflies (Rantala et al., 2000; 2010 and Rolff and Siva-Jothy, 2004). Damselflies defend themselves against ectoparasitic water mites by encapsulating the feeding tubes of the externally located mites (Rantala et al., 2010) in a similar way as they encapsulate the implant. The implant (0.25 mm diameter and 1.5 mm length) was made of nylon that was rubbed with sandpaper to provide a rough surface for the encapsulation process. Implants were made sterile in absolute ethanol. The sterile implant was inserted in the abdominal pleura on the dorsal side of the sternal-tergal margin for 7 hours to obtain a sufficient encapsulation response and to maximize the differences among individuals (Rantala et al., 2000). Animals were kept in the dark without food during the immune challenge. Control animals were treated similarly but without the insertion of the implant. Subsequently, the implant was removed and stored at -20°C until it was photographed. Adults were stored at -80°C until physiological analyses.

Each implant was photographed from three different angles under a microscope (Olympus BX51, 40× magnification) with a digital camera. The mean grey values were measured with the image analysis software Image Pro Plus. We used the mean of the grey values from the three digital pictures of each implant as a measure of the encapsulation response of that animal. The grey values of the inserted implants were corrected by subtracting the grey values from a control implant that was not inserted (Rantala et al., 2011). The highest grey values correspond to the strongest encapsulation responses.

Physiological assays

We measured the activity of PO using the protocol of Stoks et al. (2006) that was optimized for *C. scitulum*. Duplicate 20 µl aliquots of the combined supernatant of thorax and abdomen were pipetted into a 384-well microplate (Greiner 384 Flatblack). Subsequently, samples were mixed with 5 µl of chymotripsin (5 mg/ml, daily prepared) and incubated at room temperature for 5 minutes. This ensures that all pro-enzyme is converted to PO, so that we were able to measure total PO concentrations. Afterwards, we added to this mixture 15 µl L-DOPA (dihydrophenyl-l-alanine; 10 mM in cacodylate buffer, daily prepared, kept on ice and covered by an aluminum foil). Immediately after mixing with L-DOPA, the absorbance of the samples was monitored at 490 nm for 45 minutes (interval time: 40 s, 90 readings) on a temperature-controlled microplate reader at 490 nm.

Total fat content was quantified based on the method of Bligh and Dyer (1959). Twenty-five microliter of the mixture of thorax and abdomen supernatants was mixed with 175 µl H_2SO_4 (50%). This mixture was incubated at 200°C for 15 minutes. Subsequently, samples were cooled down at room temperature and duplicates of 30 µl were pipetted into a 384-well microplate (Greiner 384 Flat bottom Transparent Polystyrol, Greiner Bio-one, Germany). Afterwards, the absorbance of the samples was monitored at 340 nm. Fat contents were calculated using a standard curve of glyceryl tripalmitate and expressed as µg fat/mg adult wet mass.

Thorax protein content was quantified based of the method of Bradford (1976). One microliter of the thorax homogenate was added to 160 µl milli-Q water. Subsequently, the sample solution was mixed with 40 µl Bradford reagent. The absorbance was read at 595 nm after 5 minutes of incubation on a microplate reader. Protein content was calculated from the standard curve of bovine serum albumin.

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Supporting information S3

Table S1. The results of (generalized) linear mixed models testing for the effects of population type (Type), food level and chlorpyrifos concentration (CPF) on survival, growth rate, and the duration of the final instar of *Coenagrion scitulum* larvae. Survival data were analyzed with a binomial error structure and the logit link. Significant *P* values ($P < 0.05$) are indicated in bold.

Effect	Survival			Growth		
	df1, df2	F	*P*	df1, df2	F	*P*
Fixed effects						
Type	1, 587	0.55	0.46	1, 587	2.01	0.15
Food	1, 587	25.81	**<0.001**	1, 587	1878.34	**<0.001**
Chlorpyrifos (CPF)	1, 587	0.001	0.99	1, 587	0.38	0.54
Type × Food	1, 587	0.17	0.68	1, 587	5.71	**0.017**
Type × CPF	1, 587	0.17	0.68	1, 587	0.78	0.38
Food × CPF	1, 587	0.49	0.48	1, 587	1.00	0.32
Type × Food × CPF	1, 587	0.11	0.74	1, 587	0.08	0.78
Random effect	df	χ^2	*P*			
Population (Type)	1	5.25	0.15	2, 576	1.45	0.24

Table S2. The results of (generalized) linear mixed models testing for the effects of population type (Type), food level and chlorpyrifos concentration (CPF) on the metamorphosis success and the mass at emergence of the damselfly *Coenagrion scitulum*. Metamorphosis success was analyzed with a multinomial error structure and the cumlogit link. Significant *P* values ($P < 0.05$) are indicated in bold.

Effect	Metamorphosis success			Ma		
				Males		
	df1, df2	F	*P*	df1, df2	F	*P*
Fixed effects						
Type	1, 529	4.59	**0.032**	1, 192	2.43	0.12
Food	1, 529	24.36	**<0.001**	1, 192	118.79	**<0.001**
Chlorpyrifos (CPF)	1, 529	7.14	**0.0078**	1, 192	0.001	0.97
Type × Food	1, 529	1.12	0.29	1, 192	0.16	0.69
Type × CPF	1, 529	3.23	0.073	1, 192	0.52	0.47
Food × CPF	1, 529	0.04	0.84	1, 192	0.16	0.69
Type × Food × CPF	1, 529	0.001	0.98	1, 192	1.98	0.16
Random effect	df	χ^2	*P*			
Population (Type)	3	0.21	0.98	2, 182	0.51	0.60

Table S3. The results of general linear mixed models testing for the effects of population type (Type), food level, chlorpyrifos concentration (CPF) and the nylon monofilament insertion (Implant) on the relative flight muscle mass and fat content of males and females of the damselfly *Coenagrion scitulum*.

Effect	Relative flight muscle mass								
	Male			Female			Male		
	df1, df2	F	*P*	df1, df2	F	*P*	df1, df2	F	
Fixed effects									
Type	1, 170	3.86	0.051	1, 3.95	0.50	0.52	1, 3.22	11.40	<
Food	1, 170	16.60	**<0.001**	1, 214	92.98	**<0.001**	1, 179	25.34	<
Chlorpyrifos (CPF)	1, 170	3.00	0.085	1, 215	0.18	0.67	1, 185	0.53	
Type × Food	1, 170	0.05	0.82	1, 214	1.76	0.19	1, 179	3.81	
Type × CPF	1, 170	5.63	**0.018**	1, 215	3.18	0.076	1, 185	3.78	
Food × CPF	1, 170	2.63	0.11	1, 213	0.16	0.69	1, 185	3.27	
Type × Food × CPF	1, 170	1.69	0.20	1, 213	4.76	**0.030**	1, 185	0.36	
Implant	1, 170	0.43	0.51	1, 214	1.84	0.18	1, 180	3.21	
Type × Implant	1, 170	0.14	0.71	1, 214	2.03	0.16	1, 180	3.32	
Food × Implant	1, 170	0.07	0.79	1, 213	5.17	**0.024**	1, 185	0.65	
CPF × Implant	1, 170	0.01	0.92	1, 215	0.25	0.61	1, 185	0.19	
Type × Food × Implant	1, 170	1.72	0.19	1, 213	0.26	0.61	1, 185	0.35	
Type × CPF × Implant	1, 170	0.28	0.60	1, 215	1.66	0.20	1, 185	2.71	
Food × CPF × Implant	1, 170	0.36	0.55	1, 217	2.15	0.14	1, 185	7.57	**0**
Type × Food × CPF × Implant	1, 170	0.09	0.77	1, 217	1.64	0.20	1, 185	2.47	
Random effects									
Population (Type)	2, 168	0.61	0.55	2, 199	3.51	0.032	2, 167	2.62	

Table S4. The results of general linear mixed models testing for the effects of population type (Type), food level and chlorpyrifos concentration (CPF) on encapsulation response and phenoloxidase activity of males and females of the damselfly *Coenagrion scitulum*.

Effect	Encapsulation response								
	Male			Female			Male		
	df1, df2	F	*P*	df1, df2	F	*P*	df1, df2	F	*P*
Fixed effects									
Type	1, 94	0.09	0.77	1, 90	0.001	0.99	1, 168	4.47	**0.**
Food	1, 94	0.22	0.64	1, 90	0.01	0.91	1, 168	0.01	0
Chlorpyrifos (CPF)	1, 94	4.63	**0.034**	1, 90	0.63	0.43	1, 168	3.99	0.
Type × Food	1, 94	0.11	0.74	1, 90	0.001	0.99	1, 168	2.08	0

Type × CPF	1, 94	0.24	0.62	1, 90	0.17	0.68	1, 168	0.40	0
Food × CPF	1, 94	0.06	0.81	1, 90	0.48	0.49	1, 168	1.44	0
Type × Food × CPF	1, 94	1.61	0.21	1, 90	1.53	0.22	1, 168	1.63	0
Adult mass	1, 94	2.45	0.12	1, 90	2.87	0.093	1, 168	9.02	**0.0**
Implant							1, 168	31.42	**<0.**
Type × Implant							1, 168	0.02	0
Food × Implant							1, 168	0.54	0
CPF × Implant							1, 168	1.46	0
Type × Food × Implant							1, 168	2.05	0
Type × CPF × Implant							1, 168	4.17	**0.**
Food × CPF × Implant							1, 168	1.18	0
Type × Food × CPF × Implant							1, 168	1.19	0
Protein							1, 168	8.72	**0.0**
Random effects									
Population (Type)	2, 92	1.72	0.19	2, 88	2.19	0.12	2, 166	0.74	0

875

Appendix S4. Between-population variability within core and edge populations

The populations were coded as follows: core population 1 (Indre, France), core population 2 (Nord-Pas-de-Calais, France), edge population 1 (Saarland, Germany) and edge population 2 (Zeeland, The Netherlands).

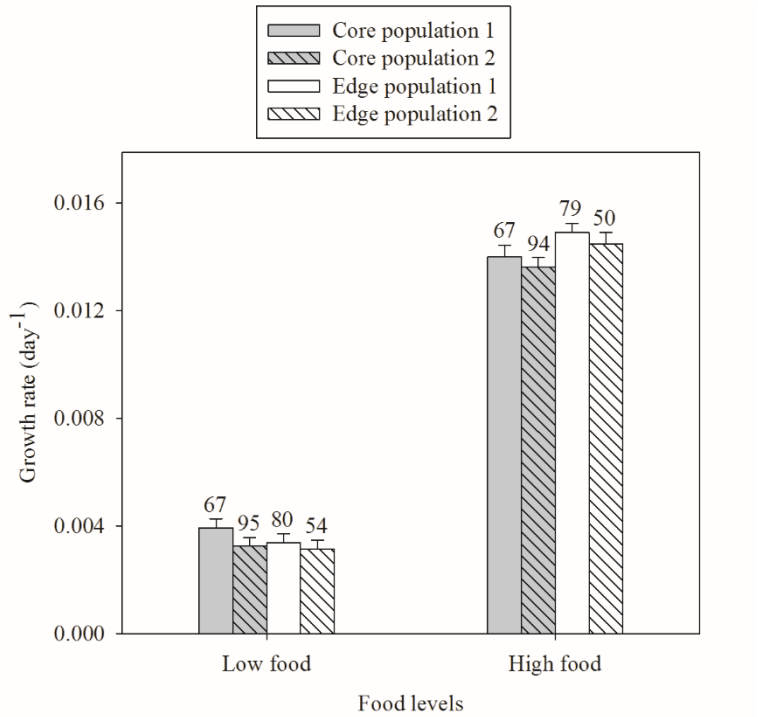

Figure S1. Between-population variability within core and edge populations for growth rate in final instar *Coenagrion scitulum* larvae as a function of food level. Least-square means are given with 1 SE. Numbers above the bars represent sample sizes.

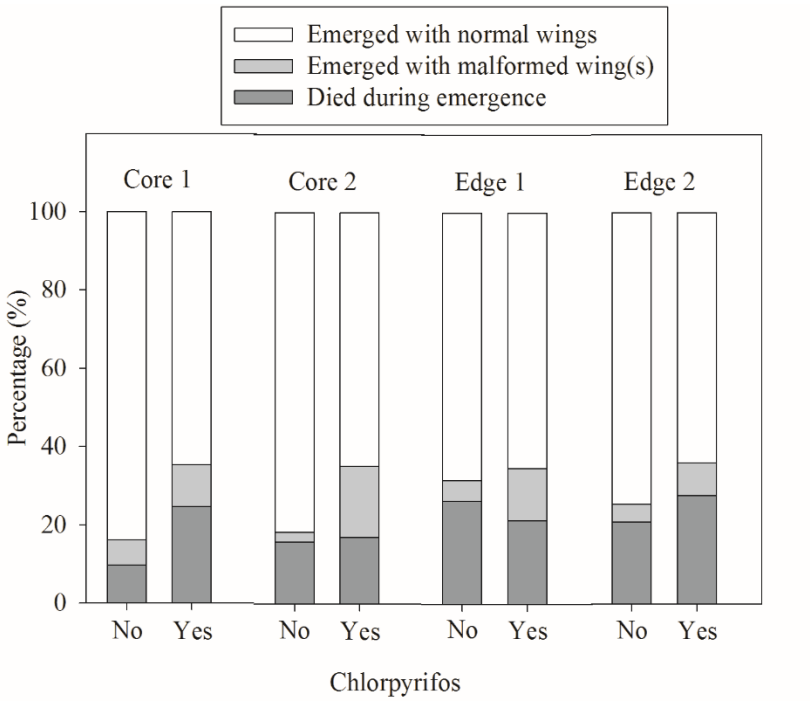

Figure S2. Between-population variability within core and edge populations for metamorphosis success of the damselfly *Coenagrion scitulum* as a function of chlorpyrifos concentration.

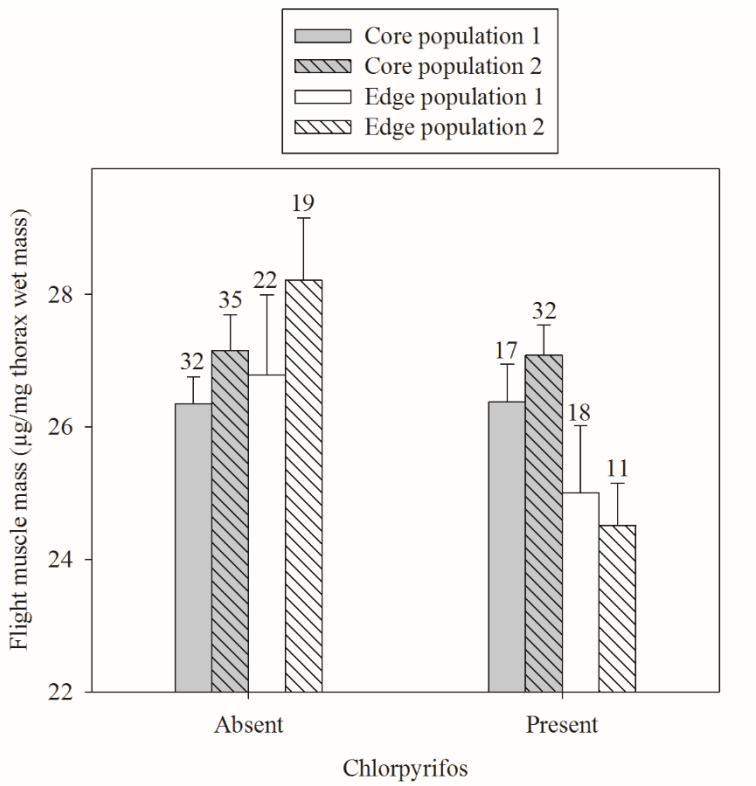

Figure S3. Between-population variability within core and edge populations for flight muscle mass of males of *Coenagrion scitulum* as a function of chlorpyrifos concentration. Least-square means are given with 1 SE. Numbers above the bars represent sample sizes.

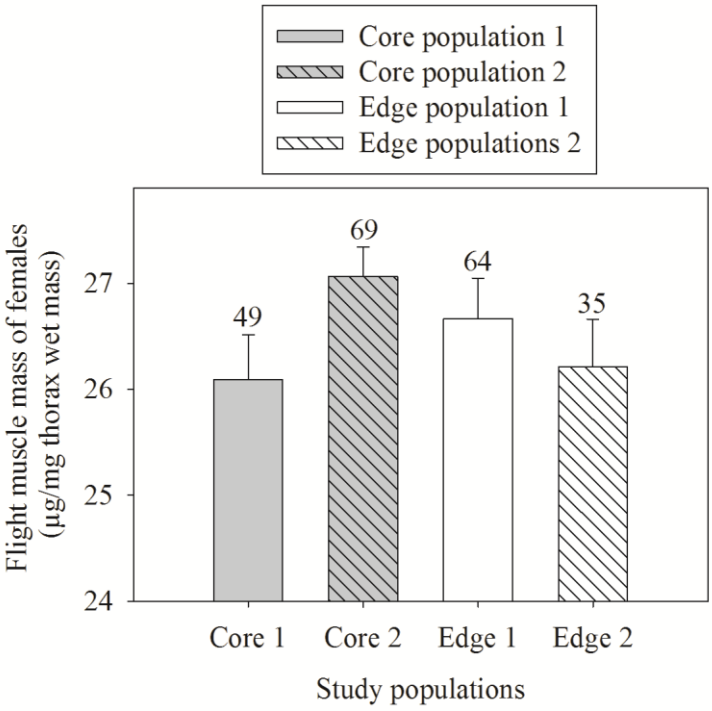

Figure S4. Between-population variability within core and edge populations for flight muscle mass of females of the damselfly *Coenagrion scitulum*. Least-square means are given with 1 SE. Numbers above the bars represent sample sizes.

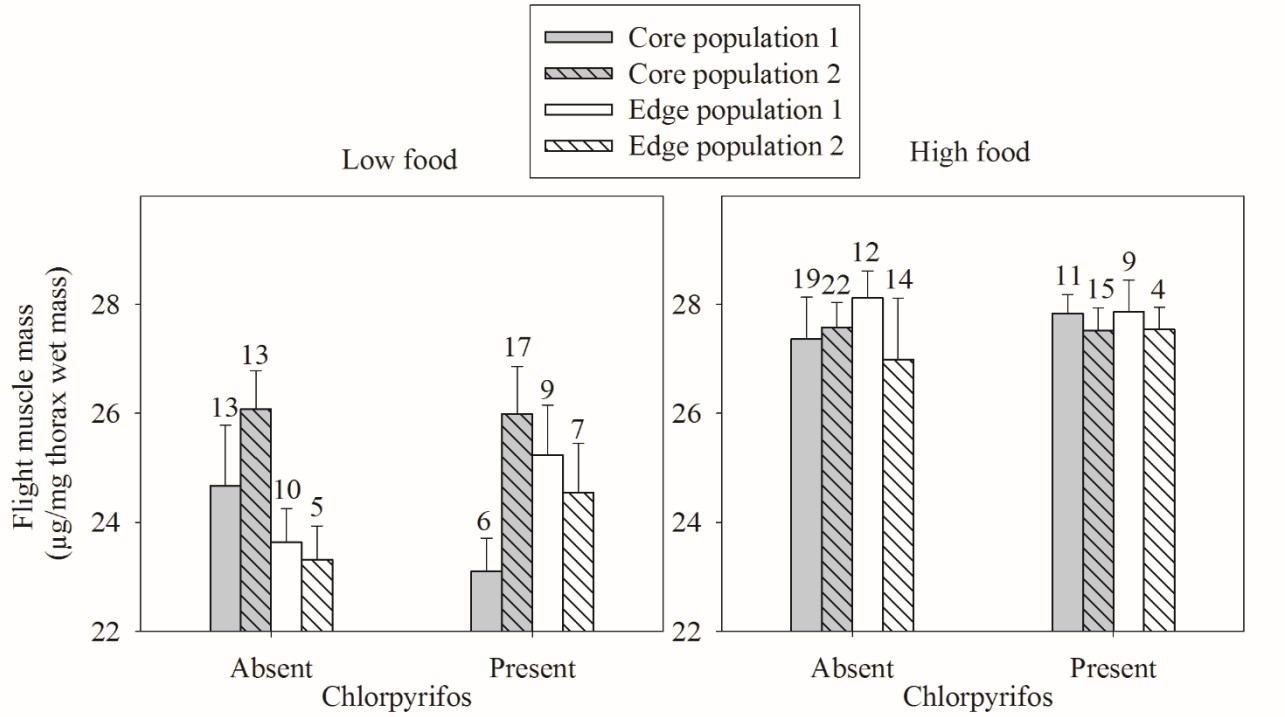

Figure S5. Between-population variability within core and edge populations for flight muscle mass of females of the damselfly *Coenagrion scitulum* as a function of chlorpyrifos concentration and food level. Least-square means are given with 1 SE. Numbers above the bars represent sample sizes.

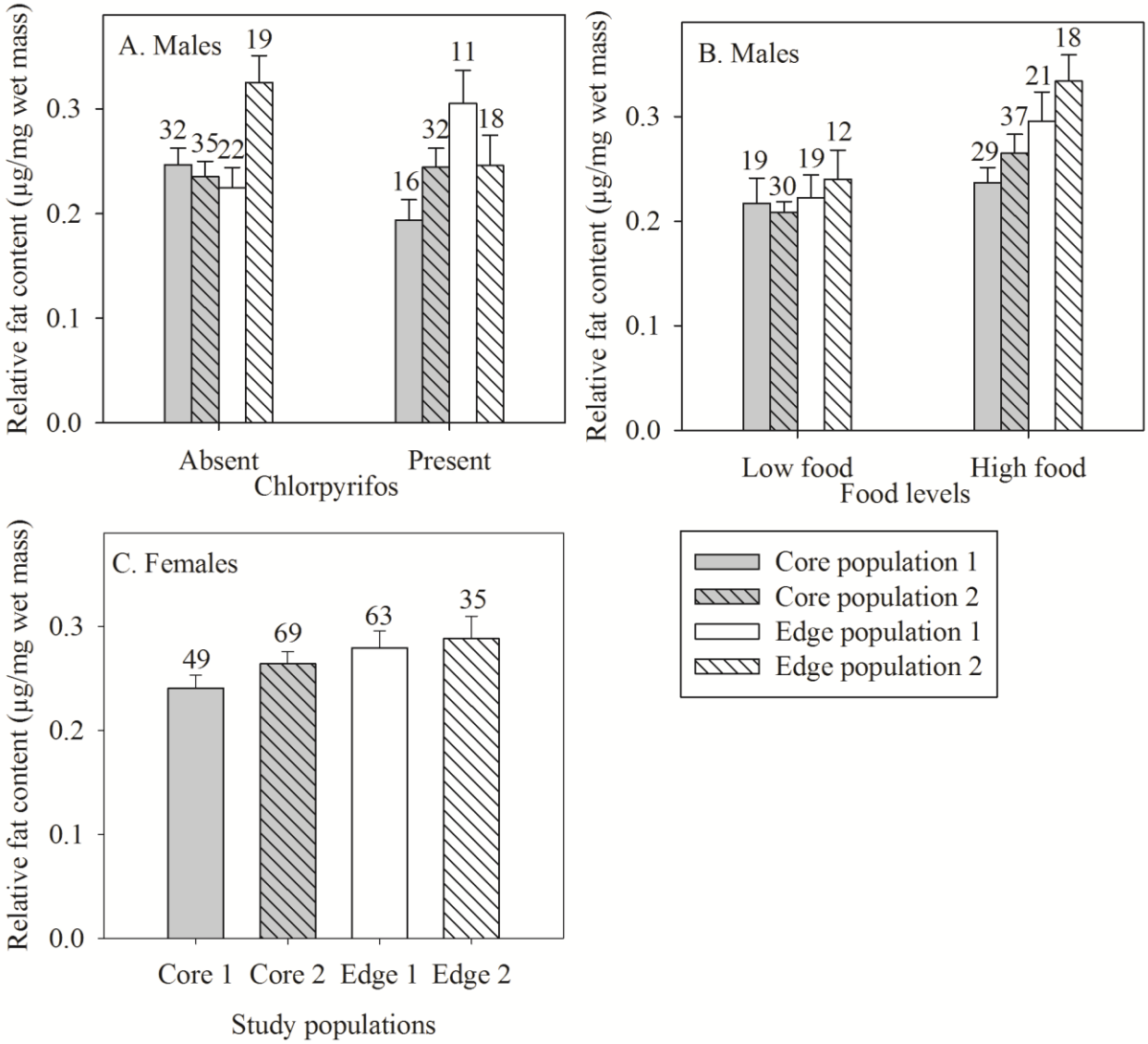

Figure S6. Between-population variability within core and edge populations for fat content of males and females of the damselfly *Coenagrion scitulum* as a function of food level and chlorpyrifos concentration (males). Least-square means are given with 1 SE. Numbers above the bars represent sample sizes.

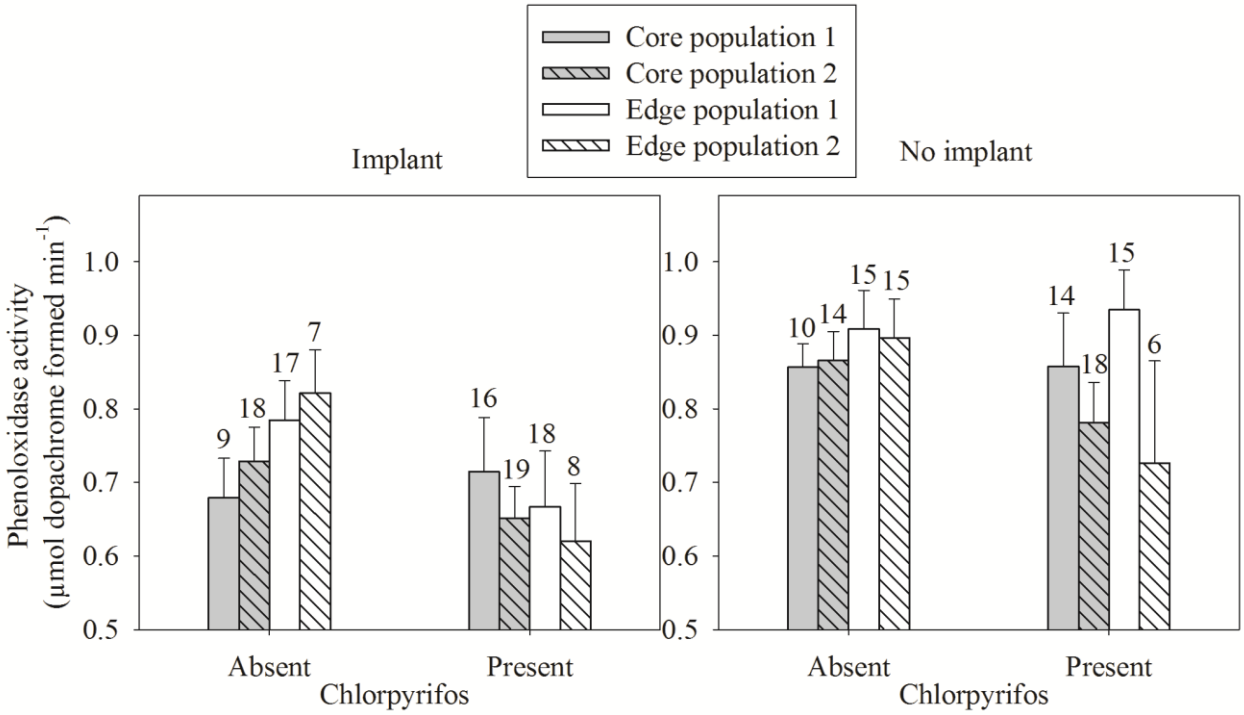

Figure S7. Between-population variability within core and edge populations for phenoloxidase activity of males of the damselfly *Coenagrion scitulum* as a function of chlorpyrifos concentration and implant treatment. Least-square means corrected for mass and total protein content are given with 1 SE. Numbers above the bars represent sample sizes.

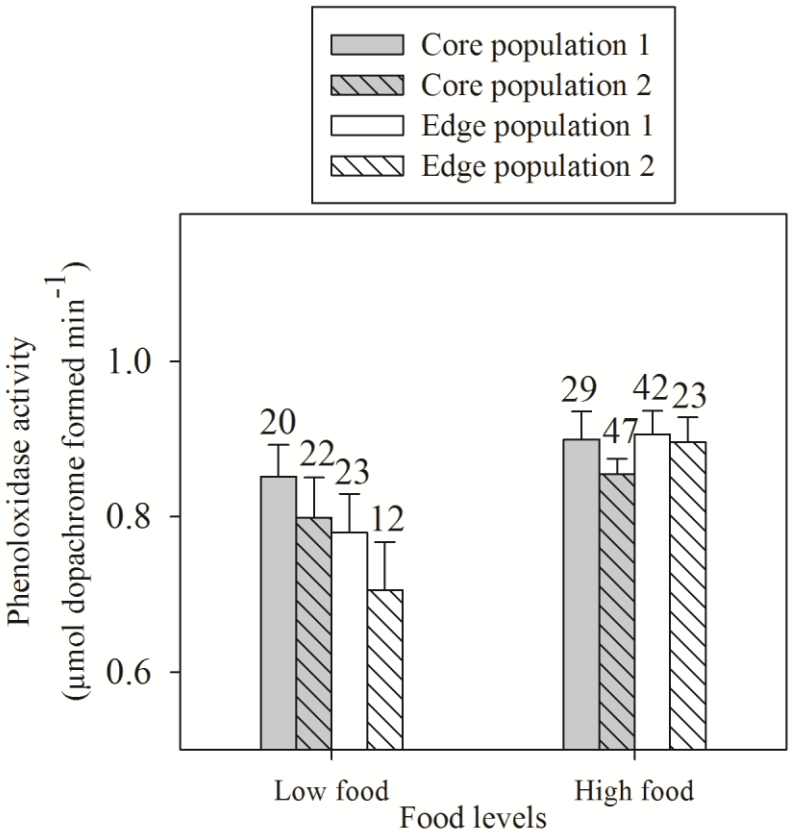

Figure S8. Between-population variability within core and edge populations for phenoloxidase activity of females of the damselfly *Coenagrion scitulum* as a function of food level. Least-square means corrected for mass and total protein content are given with 1 SE. Numbers above the bars represent sample sizes.

Appendix S5. Detailed results of flight muscle mass, fat content, encapsulation and PO activity

Flight muscle mass

There were no overall main effects of CPF exposure, population type and the nylon implant on relative flight muscle mass (Table S3, Figure S9A-D). Yet, exposure to CPF reduced the relative flight muscle mass of edge males but not of core males (Population type × CPF, $F_{1, 170} = 5.63$, $P = 0.018$, Fig. S9A-B). At low food, edge females had a lower relative flight muscle mass than core females, but only in the absence of CPF (Population type × Food × CPF interaction, $F_{1, 213} = 4.57$, $P = 0.034$, Fig. S9C-D). The insertion of the implant did not alter the relative flight muscle mass in males ($F_{1, 170} = 0.43$, $P = 0.51$), while it reduced the relative flight muscle mass in females reared at high food (Food × Implant, $F_{1, 213} = 5.17$, $P = 0.024$, Fig. S9A-D). In both sexes, the relative flight muscle mass was lower in animals reared as larvae at low food (Males: $F_{1, 170} = 16.60$, $P < 0.001$; Females: $F_{1, 214} = 92.98$, $P < 0.001$, Fig. S9A-D).

Fat content

Exposure to CPF tended to reduce the relative fat content in females ($F_{1, 200} = 3.04$, $P = 0.083$), and in core males but not in edge males (Population type × CPF, $F_{1, 185} = 3.78$, $P = 0.053$) but there was no consistent CPF effect across the two edge and the two core populations (Fig. S6A). One group of males (with low sample size), namely edge males at high food and CPF with no implant, had an unusually high fat content thereby generating a Food × CPF × Implant interaction ($F_{1, 185} = 7.57$, $P = 0.0065$) (Fig. S9E-F). In both sexes, the relative fat content was lower in adults reared as larvae at low food (Males: $F_{1, 179} = 25.34$, $P < 0.001$; Females: $F_{1, 200} = 40.45$, $P < 0.001$, Fig. S9E-H). Relative fat content tended to be lower in core females than in edge females ($F_{1, 200} = 3.25$, $P = 0.073$, Fig. S9G-H), and in core males than in edge males when reared at high food (Population type × Food, $F_{1, 179} = 3.81$, $P =$

0.052, Fig. S9E-F). The insert of an implant had no effect on the fat content of females ($F_{1, 200}$ = 0.18, P = 0.67).

Encapsulation response

Exposure to CPF in the final larval instar reduced the encapsulation response in adult males ($F_{1, 94}$ = 4.63, P = 0.034) but not in adult females ($F_{1, 90}$ = 0.63, P = 0.43, Fig. 5A). In neither sex there was a difference in the encapsulation response between core and edge populations or between food levels (all P > 0.63, Fig. S10A-B). There were no interactions between population type, food level and CPF on the encapsulation response (all P > 0.20).

PO activity

Exposure to CPF reduced the PO activity only in males of edge population when they were challenged with an implant and not in the other male treatment groups (Population type × CPF × Implant interaction ($F_{1, 168}$ = 4.17, P = 0.043, Fig. S10C, E). CPF had no effect on the PO activity of females ($F_{1, 215}$ = 0.001, P = 0.93). On average, edge males had a higher PO activity than core males ($F_{1, 168}$ = 4.47, P = 0.036); this differentiation between core and edge populations was not present in females ($F_{1, 4.51}$ = 0.06, P = 0.82, Fig. S10C-F). Food level had no effect on the PO activity (all P > 0.10). Yet, PO activity in core females was on average higher when they had been reared as larvae at low food (Population type × Food, $F_{1, 215}$ = 3.92, P = 0.049, Fig. S10D,F). The insert of an implant reduced the PO activity in both sexes (Males: $F_{1, 168}$ = 31.42, P < 0.001; Females: $F_{1, 215}$ = 17.00, P < 0.001, Fig. S10C-F).

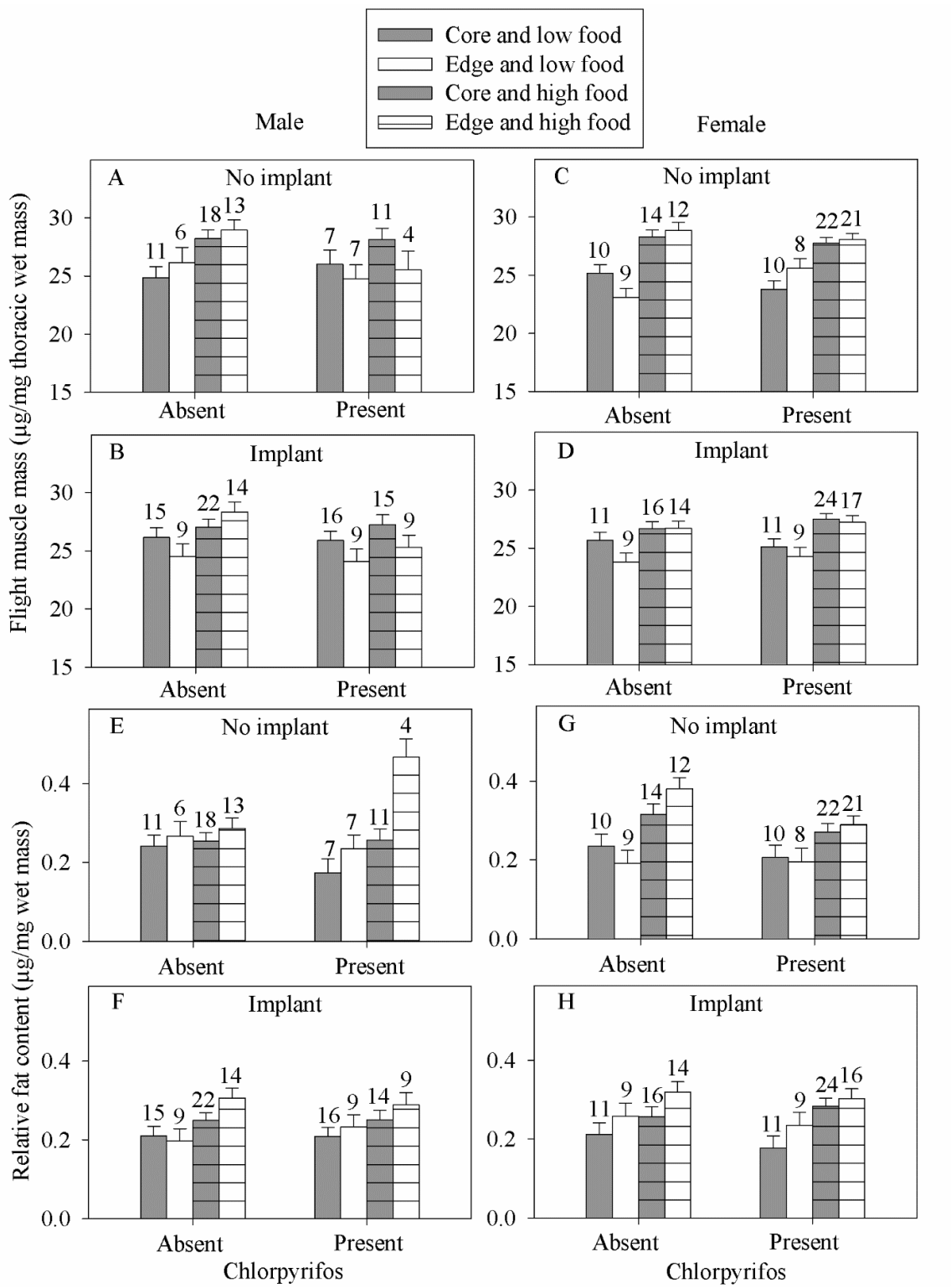

Figure S9. Mean (+ 1 SE) flight muscle mass (A-D), and total fat content (E-H) of adult *Coenagrion scitulum* as a function of chlorpyrifos concentration, food level, population type and insertion of the nylon monofilament. Numbers above the bars represent sample sizes.

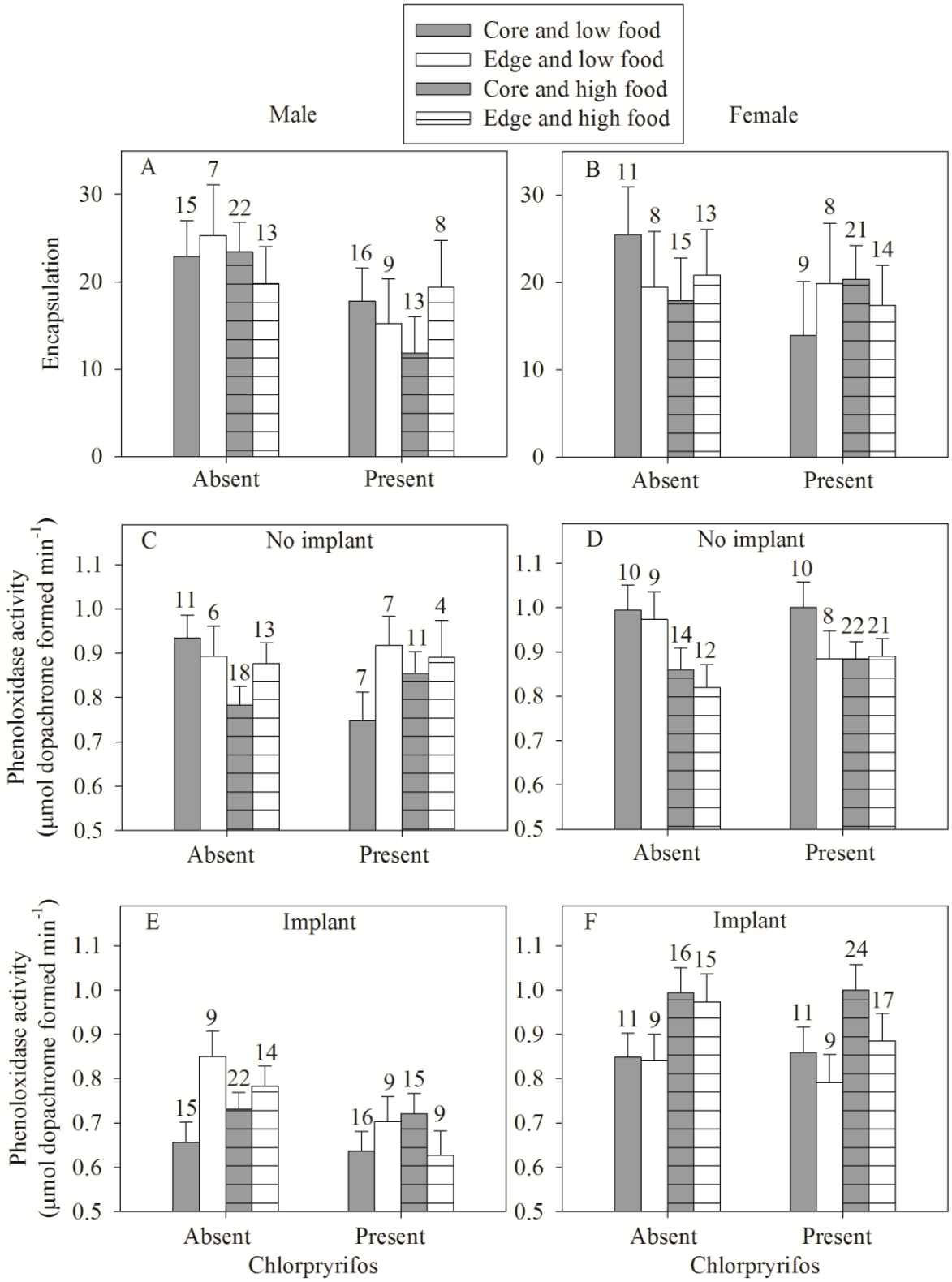

Figure S10. Mean (+ 1 SE) encapsulation response (A, B) and phenoloxidase activity (C-F) of adult *Coenagrion scitulum* as a function of chlorpyrifos concentration, food level,

population type, insertion of the nylon monofilament. Numbers above the bars represent sample sizes. Given are least-square means corrected for mass and total protein.