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Arachidonic Acid Enhances Reproduction in *Daphnia magna* and Mitigates Changes in Sex Ratios Induced by Pyriproxyfen

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Abstract

Arachidonic acid (AA) is one of only two unsaturated fatty acids retained in the ovaries of crustaceans, and an inhibitor of HR97g, a nuclear receptor expressed in adult ovaries. We hypothesized that as a key fatty acid, AA may be associated with reproduction and potentially environmental sex determination in *Daphnia*. Reproduction assays with AA indicate that it alters female/male sex ratios by increasing female production. This reproductive effect only occurred during a restricted *P. subcapitata* diet. Next, we tested whether enriching a poorer algal diet (*C. vulgaris*) with AA enhances overall reproduction and sex ratios. AA enrichment of a *C. vulgaris* diet also enhances fecundity at 1.0 and 4.0 μ M by 30–40% in the presence and absence of pyriproxyfen. This indicates that AA is crucial in reproduction regardless of environmental sex determination. Furthermore, our data indicates that *P. subcapitata* may provide a threshold concentration of AA needed for reproduction. Diet switch experiments from *P. subcapitata* to *C. vulgaris* mitigate some but not all of AA's effects when compared to a *C. vulgaris* only diet, suggesting that some AA provided by *P. subcapitata* is retained. In summary, AA supplementation increases reproduction and represses pyriproxyfen-induced environmental sex determination in *D. magna* in restricted diets. A diet rich in AA may provide protection from some reproductive toxicants such as the juvenile hormone agonist, pyriproxyfen.

Keywords

Food chain; Endocrine disruptors; Algae; Aquatic toxicology; Mixtures

INTRODUCTION

The cladoceran, *Daphnia magna* is an aquatic indicator species typically found in freshwater ponds, where they are a mid-level consumers and a food source for small fish [1]. *Daphnia* have a short maturation time and typically produce large broods of females through parthenogenesis; building rich populations in a short time span [2]. However, under stressful

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conditions such as overcrowding, scarcity of food [3], fall in ambient temperatures [4], and altered photoperiods [5], *Daphnia* may produce males [6] and switch to sexual reproduction, where a hardy ephippia (or winter egg) that can survive desiccation is produced. Methyl farnesoate, an endogenous juvenoid in *Daphnia* [6] and some juvenile hormone analog pesticides such as pyriproxyfen and fenoxycarb can induce male production [6, 7].

The polyunsaturated fatty acids (PUFAs) such as arachidonic acid (AA; n-6), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA; n-3) are important in arthropod physiology. PUFAs, especially highly unsaturated fatty acids contribute to the fluidity of cell membranes in fish and cladocerans and help these organisms to withstand cold temperatures [8]. Fish fed on zooplankton rich in PUFAs exhibit higher growth rates and fecundity [9] and zooplankton grazing on PUFA rich phytoplankton exhibit higher growth rates and fecundity [8].

Though reproduction is expensive in terms of energy expenditure [10], it is one of the principal driving forces that dictate the fitness of a species in a resource limited competitive environment. How daphnids respond and adapt to environmental cues is not clearly understood, but their efficiency in allocating limited dietary resources is crucial to survival and reproductive preferences [11]. A closer look at allocation of dietary components indicates that adult daphnids preferentially accumulate the dietary PUFAs, AA and EPA, and these two PUFAs are preferentially allocated to the ovary in the late stages of oocyte maturation [12, 13]. AA also blocks the transcriptional activity of the newly discovered group of nuclear receptors (HR97a/b/g) of which, HR97g, is highly expressed in the ovary [14]. Therefore, we considered that a diet supplemented with PUFAs and specifically AA may help repress male production as a key dietary reproductive resource.

AA is a precursor to eicosanoids that are associated with several important physiological processes such as immune function, reproduction and ion transport [15, 16]. Accumulation of AA in the ovaries of marine shrimp *Penaeus semisulcatus* is associated with oocyte maturation [17]. The prostaglandin, PGE₂, a metabolite of AA from the cyclooxygenase pathway is associated with egg laying behavior in insects [16], and PGF_{2α} is responsible for enhanced sperm motility and ovulation in mammals [18]. Therefore, accumulation of AA in the ovary is important as AA may directly influence reproduction or act through the production of eicosanoids that function as signaling molecules responsible for egg maturation or release.

Daphnia are filter feeders that preferentially consume algae as their food source. A typical daphnid diet used during environmental toxicology testing may contain *Pseudokirchneriella subcapitata*, (formerly known as *Selanastrum capricornutum*) supplemented with yeast or fish food [19, 20]. Different algal diets contain different concentrations of saturated and unsaturated fatty acids. Fatty acid concentrations have been determined in some algal species [21–23]. For example, *Nannochloropsis oculata* contains high concentrations of AA and several n-3 fatty acids. *P. subcapitata* contains moderate levels of these fatty acids, and *Chlorella vulgaris* contains high levels of linoleic acid (n-6) the precursor to AA, but little AA [23–25], and extremely low levels of most n-3 fatty acids [21, 22]. In this study, daphnids were provided algal diets that differ in fatty acid composition to help ascertain

whether high or low levels of specific unsaturated fatty acids influence reproduction and male production.

The specific influence of individual fatty acids on daphnid reproduction and their regulation of fecundity and environmental sex determination are unknown. Based on the preferential accumulation of AA in the daphnid ovary, its involvement in insect egg laying behavior and potentially its inhibition of HR97, we hypothesized that AA is associated with repressing pyriproxyfen-induced male production. The purpose of the study is to determine the influence of AA, an n-6 unsaturated fatty acid on *Daphnia* reproduction. Therefore, adult reproductively active parthenogenic *D. magna* were exposed to fatty acids, including AA alone or in combination with pyriproxyfen to determine the influence of AA on *D. magna* reproduction.

MATERIALS AND METHODS

Daphnia magna culture

A strain of *Daphnia magna* has been maintained in the Environmental Toxicology Program at Clemson University for about 20 years. Stock cultures of *D. magna* are maintained in an environmental chamber under a 16:8 hour light cycle at 21°C in moderately hard water with a diet of 6 X 10⁶ *P. subcapitata* per adult daphnid/day supplemented with 0.25 mg dry weight of blended TETRAFIN fish flakes (catalog # 46798–16140; Tetra Holding Inc., VA) in a 50 µL aqueous suspension [20].

Algal culture

Algae cultures of *P. subcapitata* were maintained in our laboratory per EPA guide lines [26]. Mother cultures of *P. subcapitata* were obtained from Aquatic Bio Systems, (Fort Collins, Colorado). Mother cultures of *N. oculata* and *C. vulgaris* were obtained from Algae Depot (Eau Claire, WI). Algae was cultured as described previously in prepared media [26] under continuous fluorescent light (Jumpstart, HydroFarms, West-Petaluma, CA, USA). All experiments were performed with *P. subcapitata* unless otherwise mentioned.

Standard acute and chronic toxicity tests

Acute toxicity of AA was determined by exposing < 24 h neonates; (4 daphnids/beaker; n=4 beakers) to AA in 40 mL of culture medium in 50 mL glass beakers. Neonates were exposed to 0.01 to 100 µM AA (>99% purity, CAS Number 506-32-1, Sigma-Aldrich, St. Louis, MO USA) dissolved in 100% ethanol.

The reproductive toxicity of AA was determined by exposing < 24h neonates (n = 12) to AA in 40mL culture medium in 50mL glass beakers using standard methods [27]. Neonates were exposed to 1.0, 2.0, or 4.0 µM arachidonic acid reconstituted in 100% ethanol for a total of 0.004% ethanol in each exposure container, including controls (untreated). Neonates were provided with the typical stock diet containing *P. subcapitata* and 0.25 mg dry weight of blended fish flakes as a 50 µL/day aqueous suspension for the 21 days of the test. The number of offspring produced during the 21 day test period was counted and reported as mean offspring/female daphnid. Neonates from the final brood were collected and a 2nd

generation chronic toxicity test was performed with the daphnids exposed multi-generationally as described previously [27] to test whether AA treatment altered reproduction in developing offspring.

Male Production Assays

Male production assays (16 days long) [28, 29] were conducted to determine the influence of specific fatty acids (palmitic acid, 99% purity, CAS Number 506-32-1, docosahexaenoic acid, 98% purity, CAS Number 6217-54-5, linoleic acid, >99% purity, CAS Number 60-33-3, all purchased from Sigma-Aldrich) in repressing male production induced by pyriproxyfen. Male production was induced by exposing the daphnids to 155 or 310 pM pyriproxyfen (50 or 100 ng/L) (>99% purity; FLUKA, Buchs, Switzerland).

Ten-day old female daphnids ($n = 10$) were exposed to fatty acids alone or in combination with pyriproxyfen while housed as described in the chronic toxicity tests. All daphnids (controls and treated groups) were exposed to 0.004% ethanol during the assays. To provide increased control over fatty acid exposure levels, daphnids were not provided fish food. Instead, daphnids were either fed the normal quantity of *P. subcapitata* (6×10^6 cells/adult daphnid) or half the normal quantity (3×10^6 cells/adult daphnid). Adult survival, the total number of neonates, the number of female neonates, and the number of male neonates produced from broods 2–5 was measured. Reproduction was determined only in neonates from broods 2–5, as fatty acid and pyriproxyfen exposure may occur after developmental sex determination in brood 1 [6, 29].

Influence of different algal diets on reproduction in adult female daphnids

Relative comparisons indicate that *Chlorella vulgaris* has low levels, and *N. oculata* has high levels of AA [21]. AA abundance approximates 0.81% of total fatty acids in *C. vulgaris*, 0.92% in *P. subcapitata* [23], and 5.8% in *N. oculata* with seasonal variances from 0.5 – 6.3% [25, 30]. Therefore, we examined the effect of each of these algal diets on survival and reproduction in 10-day old daphnids in an identical fashion as described in the male production assays. Briefly, neonates were acclimatized to their new algal diet without fish food at 6×10^6 cell/adult daphnids/day for 3 generations and then fourth generation daphnids were exposed to half the regular diet (3×10^6 cells of algae/mL/day) at ten-days old. Survival and reproduction was quantified over the next 16 days.

Male production assays with Chlorella diets

D. magna were either acclimated to a *Chlorella* diet for four generations or were switched to a *Chlorella* only diet immediately prior to pyriproxyfen treatment. Ten-day old daphnids were fed and exposed to: 1) *Chlorella* or *Chlorella* + AA, 2) *Chlorella* in combination with pyriproxyfen and AA, 3) *Chlorella* or *Chlorella* + AA immediately after switching the diet from a *P. subcapitata* based diet or 4) *Chlorella* in combination with pyriproxyfen and AA after switching from a *P. subcapitata* based diet. Survival and reproduction were monitored for the next 16 days from 4 broods (broods 2–5).

Neonatal sex determination

The sex of each neonate following pyriproxyfen exposure was assessed based on the length of the first antennae [29, 31] using a dissecting microscope (American Optical-150W haloid cold light source). Male production and overall fecundity was assessed for the first five broods, which takes approximately 12–16 days depending on the food source. However, the first brood was eliminated from the data because the presence of males in this brood is sporadic and the first brood is often exposed to pyriproxyfen after the specific developmental time frame necessary to alter the sex of the developing egg [6, 29].

Statistics

Statistical analysis of the data was performed using SAS version 9.3 with General Linear Models (GLM) procedure. Levene's test was performed to assess the equal variance assumption among treatment groups. Shapiro Wilks test was applied to the residuals of the model fit for males, females and total number of offsprings produced to assess normality of the data. In addition, the data was further analyzed using Generalized Linear Mixed (GLIMMIX) Model assuming Poisson distribution for the purpose of comparing the final conclusion between the two models. The statistical significance among the treatment groups are typically assessed by one-way ANOVA followed by Studentized Tukey's Range Test at $p < 0.05$ in GLM except when statistical differences were determined only by one-way ANOVA (GraphPad Prism Version 4.3; GraphPad Software La Jolla CA, USA) followed by Tukey's multiple comparison tests. Differences in survival were determined using Fisher's exact test (<http://www.vassarstats.net/>). The LC50 of arachidonic acid was determined as described previously using the sigmoidal dose-response curve function of GraphPad Prism 4.3 [32].

RESULTS

Toxicity assessment of arachidonic acid following acute and chronic exposure

To determine future exposure concentrations, AA's acute and chronic toxicity were evaluated using standard methods. Neonatal *D. magna* exposed to AA show no acute toxicity until treated with concentrations of at least 10 μM (Supplemental Data; Table S1; $\text{LC}_{50} = 7.6\mu\text{M}$). AA's reproductive toxicity was determined using standard chronic toxicity assays and AA did not adversely impact any of the reproductive endpoints measured at any of the concentrations examined (up to 4 μM ; Supplemental Data, Table S1). Therefore, further studies were performed with 1 or 4 μM AA.

Can fatty acids alter sex ratios in the presence of pyriproxyfen?

Male production is associated with a number of factors and we hypothesized that poor diet or more specifically a diet full of healthy unsaturated fatty acids may repress male production, in part because AA and other UFAs are retained in the ovaries [17]. Therefore, we exposed daphnids to the juvenile hormone analog (JHA), pyriproxyfen (155 pM; 50 ng/L) in conjunction with several fatty acids under normal feeding conditions and dietary restriction (half the normal food). Toxicity was minimal as most fatty acids do not elicit toxicity until concentrations reach about 10 μM (unpublished data), and toxicity only

occurred in the groups co-treated with both pyriproxyfen and linoleic acid (Supplemental Data, Table 2). The number of males and females produced over the next 10 days was also quantified. Under a typical *P. subcapitata* laboratory diet (6×10^6 cells/adult daphnid/day), none of the fatty acids at 4 μM concentrations altered pyriproxyfen-induced male production (Figure 1A). When the diet was reduced to half the regular quantity of *P. subcapitata*, 4.0 μM AA increased the number of females produced. Male production was not affected. None of the other fatty acids significantly affected overall reproduction, or male or female production, during pyriproxyfen exposure (Figure 1B).

AA alters pyriproxyfen-mediated neonatal sex ratio

Because AA was the only fatty acid that increased female production, the role of AA as a potential inhibitor of pyriproxyfen-mediated male production was verified. A male production assay was performed exposing reproductively mature *D. magna* to two different environmentally relevant concentrations of pyriproxyfen (155 or 310 pM) [29] and AA (1.0 or 4.0 μM) either alone or in combination while being fed $\frac{1}{2}$ the typical number of *P. subcapitata* cells/day. The *Daphnia* exposed to AA alone did not show any significant changes in the total number of neonates produced when compared to untreated *Daphnia* (Figure 2A). *Daphnia* that were only exposed to pyriproxyfen exhibited a dose-dependent decrease in overall fecundity and a corresponding increase in the number of male neonates produced (Figure 2B). AA exposure only decreased male production when *Daphnia* were co-exposed to the lowest concentration of pyriproxyfen and the highest concentration of AA (Figure 2C). More importantly, AA significantly increased female production in a concentration-dependent manner and increased overall reproduction when *Daphnia* were co-exposed with pyriproxyfen (Figure 2C/D) confirming the previous experiment.

Daphnids co-exposed to pyriproxyfen and AA show a decrease in the male/female sex ratio compared to daphnids only exposed to pyriproxyfen, primarily because of a significant increase in female production in the co-exposed daphnids. The increased female production also accounts for the increase in fecundity relative to daphnids exposed only to 310 pM pyriproxyfen, suggesting AA provides recovery or protection from pyriproxyfen induced repression of *Daphnia* reproduction [29] and male producing effects. However, at 155 pM pyriproxyfen, AA reduced male production and increased female production but without an overall increase in reproduction. This may be due to differential effects by the different pyriproxyfen concentrations, or may in part be due to less repression of reproduction by the lower concentration of pyriproxyfen (Figure 2CD). In summary, AA altered the male/female sex ratio in pyriproxyfen-exposed daphnids primarily but not exclusively due to an increase in female neonate production.

Influence of algal diet on reproduction

Because AA enrichment resulted in changes in the male/female sex ratios following pyriproxyfen exposure and increased fecundity at a concentration of pyriproxyfen (310 pM) that represses overall reproduction, we investigated whether an algal diet low in AA represses reproduction in *D. magna*. *D. magna* were acclimatized to new dietary conditions without fish food and with different algae species that vary in AA concentration (*N. oculata* > *P. subcapitata* > *C. vulgaris*) for three full generations. The number of neonates produced

by adult 4th generation *D. magna* was quantified. A *C. vulgaris* diet, which is poor in AA, resulted in a significant decrease in the number of offspring/adult compared to the two diets richer in AA (*N. oculata* and *P. subcapitata*) (Figure 3).

Influence of AA enrichment of an AA poor diet on fecundity and neonatal sex ratios

Therefore, we examined the influence of AA supplementation on fecundity and male production with a *C. vulgaris* diet. Daphnids fed a *C. vulgaris* diet for four generations were exposed to pyriproxyfen, AA, or pyriproxyfen and AA. As observed in Figure 3, the daphnids did not reproduce as well when fed only *C. vulgaris*. Reduced health was also manifested as lethality as 2 out of 12 daphnids died shortly after the initial co-exposures with both AA and pyriproxyfen. Other treatments did not show such toxicity and continued exposure to the AA-pyriproxyfen mixture did not cause further toxicity (Supplemental Data, Table S2). AA increased overall fecundity in a dose-dependent manner (Figure 4) with or without pyriproxyfen present (52–95%) (Figure 4AB). AA also increased the number of males produced in a dose-dependent manner (45%) when co-treated with pyriproxyfen, but not the number of females as observed following the ½ *P. subcapitata* diet (Figure 2).

Concurrently, a diet switch experiment was performed in which *D. magna* fed *P. subcapitata* until 9-days old were switched to the AA poor *C. vulgaris* diet at the onset of the pyriproxyfen and AA exposures. The purpose of this treatment was to forego any acclimation to *C. vulgaris* and a low AA diet that may occur in the daphnids fed only *C. vulgaris*. Once again AA increased fecundity in the AA only and pyriproxyfen + AA groups in a dose-dependent manner (55% and 90%, respectively) (Figure 4CD). However, during the diet switch experiments female production was significantly altered in the co-exposed group, but not male production. Thus, the diet switch experiment in which daphnids are fed *P. subcapitata* as neonates and *C. vulgaris* as adults is consistent with the previous experiment with *P. subcapitata* (Figure 2), but not the experiment following acclimation to *C. vulgaris* (Figure 4B), suggesting dietary AA sequestration following *P. subcapitata* feeding.

DISCUSSION

Arachidonic acid enhances reproduction in dietary-restricted *Daphnia magna*. AA also increases female/male sex ratios in the presence of the JHA pesticide, pyriproxyfen. *Daphnia* accumulate AA from their phytoplankton diet and retain it in the ovaries [12, 33] and eggs [13]. The retention of AA is probably crucial in their reproductive physiology; especially under restrictive or fluctuating dietary conditions as AA supplementation of an abundant diet of *P. subcapitata* did not increase reproduction or alter pyriproxyfen-mediated male production. However, AA supplementation of a ½ *P. subcapitata* restricted diet or *C. vulgaris* diet increased fecundity.

For example, *P. subcapitata* is part of a typical laboratory daphnid diet and AA supplementation significantly improves reproduction in only a restricted *P. subcapitata* diet. *C. vulgaris* lack AA and daphnids fed a diet of *C. vulgaris* show significantly lower fecundity even when fed 6×10^6 cells/adult (Figure 3). Supplementation of AA to daphnids cultured for 4 generations on a *C. vulgaris* diet significantly improved fecundity (Figure 4AB) with or without pyriproxyfen present. In fact, the overall increase in fecundity was

nearly the same with or without pyriproxyfen (60 to 90% Figure 4AB). *N. oculata* and *P. subcapitata* algal diets with significantly greater AA concentrations (especially *N. oculata*) had nearly equivalent fecundity [30]. This suggests that these algal diets contain the necessary nutrients, including AA to support reproduction, but restricted diets or a diet rich in *C. vulgaris* may not contain the necessary AA to support maximal reproduction.

Diet switch experiments were performed so that daphnids did not have time to acclimate to a diet lacking in AA (*C. vulgaris*). When reproductive age daphnids were switched from *P. subcapitata* to *C. vulgaris*, AA increased fecundity 55% (Figure 4C), which is close to the 60–90% fecundity increases measured earlier (Figure 4AB). Typically, following pyriproxyfen-exposure, increased female production is the primary driver of increased fecundity when daphnids are co-exposed to AA with a *P. subcapitata* diet. Increased male production is the primary driver of increased fecundity following AA exposure with a *C. vulgaris* diet (Figure 2, 4). During a diet switch from *P. subcapitata* to *C. vulgaris*, AA caused a concentration-dependent increase in fecundity that initially was driven by increased male production, however at 4.0 μM AA, was driven by increased female production during the pyriproxyfen co-exposures (Figure 4D). AA is one of only two UFAs retained in daphnids (the other is EPA)[12, 34], and its retention during the initial *P. subcapitata* diet before the switch to *C. vulgaris* may allow daphnids to accumulate some EPA and AA and in turn acclimate to specific environmental and dietary challenges in a changing pond ecosystem or during algal succession. In addition, fecundity was not increased as much in the diet switch experiment without pyriproxyfen possibly because of greater reproduction in the controls fed the *P. subcapitata* diet early in their lifecycle (Figure 4AC). Furthermore, early lifecycle exposure to *P. subcapitata* during the co-exposures to 4 μM AA + pyriproxyfen also manifested itself by providing some protection from male production (Figure 4D). Taken together, this data suggests that AA enhances reproduction, provides better fitness in the presence of pyriproxyfen, and a better diet early in life has some carry over to reproduction later in life.

There are other differences between the diets besides AA levels. *P. subcapitata* have close percentages of total fatty acids, but greater percentages of mono and polyunsaturated fatty acids compared to *C. vulgaris* [35]. *N. oculata* also has significantly greater unsaturated fatty acid content than *C. vulgaris* or *P. subcapitata* [25]. These other fatty acids may also explain response differences. All of the culture conditions were the same for culturing the algae and in turn carbon:nitrogen:phosphorus (C:N:P) ratios were most likely quite similar as these ratios are often correlated with the media in which they grow (such as streams/lakes) [36]. As such marine and laboratory cultured algae have very stable C:N:P ratios (Redfield ratio), while streams and lakes show more variability. The two variables most associated with growth and reproduction in *Daphnia* are n-3 fatty acid and phosphorus limitations and the studies vary in the outcome depending on how much P is limited [37, 38]. Increased growth rates caused by 24 h light may increase C content [39]. This may increase C-ratios and make P-limiting. *C. vulgaris* has a high growth rate relative to the other *Chlorophyceae* algae used in this study (*P. subcapitata*) and in turn reduce P concentrations and the growth potential of *C. vulgaris*. Other studies have found *C. vulgaris* a poor diet in *Daphnia*, and partially attributed this to their heavy cell walls [40]. Overall, there are several dietary factors of algae that can influence daphnid growth and these factors may play a role in observed reproductive

differences in daphnids. Therefore, AA is clearly not the only potential limiting factor within these diets. However, supplementation of both *C. vulgaris* and ½ fed *P. subcapitata* improved reproduction in the presence of AA regardless of diet.

Our studies also demonstrate the importance of a healthy diet for a proper response to toxicant exposure. AA supplementation clearly helped support reproduction in the presence of the JHA pesticide, pyriproxyfen. Pyriproxyfen significantly reduces fecundity (Figure 2) [29]. Supplementation of *C. vulgaris* or ½ *P. subcapitata* with AA (1.0 or 4.0µM) prompted a recovery caused by the pyriproxyfen-induced (155 pM or 310 pM pyriproxyfen) drop in fecundity (Figure 2BD, Figure 4BD). AA supplementation overcomes this repression of fecundity when diets are restricted either by the number of algae (1/2 *P. subcapitata*) or the source of algae (*C. vulgaris*). Diets are not restricted during environmental toxicology tests; however, algal restricted diets may be common in ponds, especially prior to summer algal blooms. Furthermore, algal diets may not be ideal because of the growth rate or composition of the algae in the pond or lake [24, 41, 42]. Therefore, pyriproxyfen may have greater effects than expected under some specific adverse conditions depending on the algae present during that season.

Fatty acid composition of *Daphnia* primarily matches their dietary fatty acid composition [43], except for the retained fatty acids, AA and EPA. AA is specifically retained in the ovaries of *Daphnia* [34], and investigators have hypothesized that AA is crucial in daphnid reproduction with a recent publication demonstrating a role for AA in *D. magna* reproduction with mixed benefits in the presence of a parasite [44]. In addition, previous studies demonstrated that dietary AA concentrations enhance percent females per spawn, number of spawnings, eggs per female and promotes egg development in the marine shrimp, *P. monodon* [45]. However, another recent study indicated that AA is not as crucial for reproduction of *Daphnia pulex* compared to EPA [46]. Ravet et al, [46] indicate surprise with their results and also suggest that a different diet may produce significantly different results. AA was supplemented in this study; however, and AA concentrations were not completely restricted in the controls. Based on our data, we hypothesize that there is an AA threshold concentration in the daphnid ovary and a drop in ovarian AA levels below the threshold concentration must occur before perturbations in reproduction are evident. Based on our results, we hypothesize that the algal diet used and the amount of algae fed to each adult could significantly alter the results.

Interestingly, AA inhibits all three recently discovered *D. magna* HR97 receptors (HR97a/b/g) [14]. The HR97 (NR1L) receptors are related to the HR96 group (NR1J) of receptors [47] that are promiscuous xenobiotic, cholesterol, triacylglycerol, and fatty acid receptors found in *Daphnia* and *Drosophila* and related to the mammalian receptors, CAR, PXR, and VDR [32, 48, 49]. The concentration of AA needed to inhibit the *D. magna* HR97 receptors is high (EC50s of 25–200µM) [14]; however, it has been reported that AA concentrations can reach up to 35 – 100 µM under inflammatory or active conditions in humans [50]. Consequently, AA could reach the concentrations necessary to inhibit HR97g or HR97a, the most AA-sensitive of the *D. magna* HR97 receptors with EC50s of 26.8 and 93.8 µM [14], and modulate reproduction.

AA's diverse effects on sex ratios depending on diet are interesting and may be caused by one or more pathways modulated by AA. HR97g is also weakly activated by pyriproxyfen at supraphysiological concentrations in vitro [14]. Conceivably AA could directly inhibit pyriproxyfen's actions on the HR97 receptors. However, we consider it unlikely that pyriproxyfen works directly through HR97g as pyriproxyfen activates HR97g only at supraphysiological concentrations in vitro and may not reach these concentrations in vivo. Furthermore, a JHA receptor associated with male production in daphnids, MfR was recently discovered independently by two groups [51, 52].

Doublesex directs sexual determination in individual daphnids [53], and AA could inhibit pyriproxyfen-mediated induction of doublesex. However, AA also increases reproduction under dietary restricted conditions in which pyriproxyfen is not present. Therefore, we consider it more likely that AA is not directly involved in sexual reproduction in daphnids, but is retained in the ovaries as a key UFA for reproduction. In turn, AA increases fecundity under many circumstances, including adverse circumstances in which daphnids are exposed to pyriproxyfen, because AA is a key signaling molecule and resource for reproduction. Further, we hypothesize that AA is retained in *Daphnia* so that it is maintained at or above reproductive threshold concentrations in the ovaries.

In summary, AA supplementation enhanced fecundity compared to similarly treated daphnids during a restricted diet. In addition, AA supplementation enhanced fecundity and female/male ratios under the stress of JHA exposure during restricted diets or low AA diets, indicating the toxicant's adverse hormonal effects were mitigated by AA. Overall, pyriproxyfen is more toxic when dietary resources were limited as compared to a diet complete with supplemented AA. This indicates that diet is crucial in recovery or acclimation to some toxicants as AA supplementation significantly improved fecundity in control daphnids and daphnids exposed to the JHA pesticide, pyriproxyfen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Arachidonic acid alters pyriproxyfen-mediated male/female offspring ratio
 Reproductively mature *Daphnia magna* were exposed to 155 pM pyriproxyfen or pyriproxyfen and several different dietary fatty acids at 4.0μM; arachidonic acid (AA), Docosahexaenoic Acid (DHA), Palmitic Acid (PA) and Linoleic Acid (LA). Production of male and female offspring was quantified from broods 2–5 during the next 10 days. (A) *D. magna* fed at normal levels of *P.subcapitata* (6×10^6 cells/adult) (B) *D. magna* fed at half the normal level of *P.subcapitata* (3×10^6 cells/adult). White bars represent females, gray bars males, and combined they represent the total number of offspring. The superscript (T) stands for total, (M) for male and (F) for number of female offsprings produced. Groups with superscripts T, M or F with the same letters (a/b) are not statistically different. Groups with different alphabets indicate statistical differences as indicated by one-way ANOVA using a General Linear Model (GLM- SAS version 9.3) followed by Tukey's studentized Range test at $p < 0.05$. Data are expressed as Mean $\pm$ SEM.

(GLM-SAS.9.3 version) followed by Tukey's studentized Range test at $p < 0.05$. Data are expressed as Mean $\pm$ SEM.

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Figure 3. Influence of algal diet with varying levels of arachidonic acid on *Daphnia* reproduction *Daphnia magna* were acclimatized to algal diets for four generations. Influence of diets that differ in AA content was quantified based on total neonates produced by reproductively mature adults. An * indicates a significant difference in the total number of neonates produced as determined by one-way ANOVA followed by Dunnett's multiple comparison test (* = $p < 0.01$). Data are expressed as Mean $\pm$ SEM.

Figure 4. Reproductive effects of supplementing an arachidonic acid poor diet (*Chlorella vulgaris*) with AA in diet-acclimatized and diet-switched daphnids
 Reproductively mature *Daphnia magna* were exposed to 155 pM pyriproxyfen, or 1.0μM or 4.0μM AA alone or in combination. Production of male and female offsprings was quantified (A) *D. magna* acclimatized to *C. vulgaris* for 4 generations and exposed to AA. (B) *D. magna* acclimatized to *C. vulgaris* for 4 generations and exposed to pyriproxyfen or a combination of pyriproxyfen and AA. (C) *D. magna* cultured with *P. subcapitata* and diet-switched to a *C. vulgaris* supplemented with 1.0μM or 4.0μM AA at 10-days old (D) *D. magna* cultured with *P. subcapitata* and diet-switched to a *C. vulgaris* and supplemented with 0, 1.0 or 4.0μM AA during pyriproxyfen-treatment at 10-days old. (CV) *Chlorella vulgaris*; (U) Untreated; (A) Arachidonic acid; (P) Pyriproxyfen-155pM (PSC) *Pseudokirchneriella subcapitata*; (PSC-CV-U) *P. subcapitata* switched to *C. vulgaris* untreated; (PSC-CV-A) *P. subcapitata* switched to *C. vulgaris* with arachidonic acid; (PSC-CV-P) *P. subcapitata* switched to *C. vulgaris* with pyriproxyfen; (PSC-CV-AP) *P. subcapitata* switched to *C. vulgaris* with arachidonic acid and pyriproxyfen. White bars represent females, gray bars males, and combined they represent the total number of offspring. The superscript (T) stands

for total, (M) for male and (F) for number of female offspring produced. Groups with superscripts T, M or F with same letter (a/b) are not statistically different. Groups with superscripts with different letters indicate statistical differences as determined by one-way ANOVA using General Linear Model (GLM- SAS.9.3 version) followed by Tukey's studentized Range test at $p < 0.05$. Data are expressed as Mean $\pm$ SEM.