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Effects of insect growth regulators on the mosquito-parasitic nematode *Romanomermis iyengari*

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Abstract Pyriproxyfen, a juvenile hormone analogue, diflubenzuron, a chitin synthesis inhibitor, and azadirachtin, an ecdysone agonist, are three insect growth regulators (IGRs) considered as selective and effective insecticides for mosquitoes. *Romanomermis iyengari* (Welch) is a mosquito-parasitic mermithid that can provide biological control against many medically important mosquito species. The compatibility of these two control tactics was tested by evaluating the sublethal effects of exposure to IGR on nematode developmental stages (preparasitic, parasitic, and preparasitic + parasitic) using *Culex pipiens* larvae as the host. Sublethal concentrations of IGRs were 90 % emergence inhibition of host mosquito. Preparasitic exposure to pyriproxyfen, azadirachtin, and diflurbenzuron had no effect on infectivity, parasite load, sex ratio, or male size but reduced nematode female length and increased male sex ratio at one parasite/larva. When IGRs treatments were made against the parasitic and preparasitic + parasitic stages, pyriproxyfen and azadirachtin reduced *R. iyengari* infectivity, parasite load, and male nematode length, whereas pyriproxyfen exposure increased male sex ratio and reduced the female *R. iyengari* length. Thus, IGRs have significant negative impacts on different stages of mosquito mermithid that can destabilize the balance of host–parasite population interaction. Therefore, IGRs should be used with caution in mosquito habitats where these parasites have established.

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Introduction

Romanomermis iyengari (Welch) is an aquatic mermithid nematode that is a host-specific parasite of mosquitoes (Platzer 2007). The mermithid life cycle centers on the infective or preparasitic stage, a second-stage juvenile which enters the mosquito hemocoel by penetrating the host cuticle. Here, the parasitic stage develops and, after depleting host nutrients, kills the host by emerging as a postparasitic stage. Postparasitic nematodes fall to the bottom of mosquito pools where they molt twice to become adults and mate. Females lay their eggs in the soil substratum of the mosquito's larval habitat.

Host mortality and reports of extensive natural infection have driven interest in mosquito mermithids in biological control (Platzer 2007). *R. iyengari* has shown particular promise in field efficacy trials. In Cuba, Santamarina et al. (1993) reported infection rates of 75 to 100 % against medically important anophelines and culicines when applied at 1,000 preparasites/m^2. In subsequent field experiments in Mexico and Brazil, Santamarina et al. (1999) achieved similar infection rates, ranging from 85 to 97 % after 1 week, at 2,000–3,000 preparasites/m^2 against 12 anopheline populations. Nevertheless, mermithids as biological insecticides that is as inundative agents for short-term control remain unrealized, most notably because mass culture requires expensive in vivo rearing methods (Petersen and Willis 1970; Gordon 1981). There will be minimal interest in developing mosquito mermithids as biological insecticides against mosquitoes while the microbial biopesticide *Bacillus thuringiensis* var. *israelensis* remains a widely available, inexpensive, and effective alternative biological agent (WHO 2006).

Whatever limitations mosquito mermithids have as biological insecticides, there is little doubt that they are important natural biological control agents (Platzer 2007). There is concern, however, that conventional insecticides applied to mosquito larval habitats may negatively impact natural mermithid populations, thereby reducing their biological control usefulness. The insect growth regulators (IGRs) are considered to be safe for nontarget organisms because they mainly interfere to the insect development via unbalancing the endocrine system or weaken the chitinous structure (Mulla et al. 1986). World Health Organization (WHO 2006) recommended various IGRs for larval mosquito control belonging to juvenile hormone analog (JHA) and chitin synthesis inhibitor, and these IGRs are categorized as unlikely to pose an acute hazard in normal use. *Romanomermis culicivorax* (Ross and Smith) has been tested for compatibility with an IGR. Laboratory studies by Finney et al. (1977) and Levy and Miller (1977) reported that development of *R. culicivorax* was not affected by the IGR Altosid ®, one of the first commercially available JHA. These authors limited their treatments to the free-living preparasitic and postparasitic stages and did not test against the parasitic stages within the host. The absence of JHA impact on the free-living stages was attributed to nematodes lacking a neuroendocrine system for a JHA to bind onto (Gordon et al. 1996). These studies suggested more extensive research examining IGRs impact on mermithid nematodes.

In developing integrated mosquito control programs involving mermithid nematodes and insecticides, we must know the impact that the chemicals may have on the parasite (Gordon et al. 1996). The effects of IGRs on *R. iyengari* have not been tested. We hypothesize that IGRs exposure to preparasitic, parasitic, and both stages of *R. iyengari* negatively impact the biological parameters that will destabilize the host–parasite population interactions. We tested this by exposure of mermithid developmental stages to 90 % emergence inhibition concentration of three IGRs to the host mosquito, each with a different mode of action. Diflubenzuron is a chitin synthesis inhibitor widely used for control of dipteran pest insects. Pyriproxyfen is a JHA highly active against a wide range of insects including many medically important species. Azadirachtin is an ecdysone agonist that causes developmental abnormalities in insect.

Materials and methods

Culture of *Culex pipiens pipiens* L.

Larvae of *C. pipiens pipiens* for bioassays were obtained from a colony established from eggs collected from East Brunswick, NJ, USA 20 years ago. The colony was maintained at 26±2 °C and a relative humidity of 75±5 % with a 16:8 L/D photoperiod. Adults were held in 30×30×30 cm aluminum screen cages and supplied with 10 % sucrose solution on cotton wicks. Restrained quails were used to blood feed females (Rutgers University Animal Use Protocol #86-129). Subsequent egg rafts were hatched and larvae were held in enamel trays with 1 l of deionized water and 0.15 g of brewer's yeast. Second instars were used in all bioassays.

R. iyengari culture

All tests were conducted with *R. iyengari* reared on larvae of *C. pipiens pipiens* according to Petersen and Willis (1972). Preparasitic nematodes were obtained for tests by flooding eggs 12 h prior to testing. A host/parasite infection ratio of 1:5 at 100 preparasitic nematodes/ml was used in all tests.

Insect growth regulators

Insect growth regulators evaluated were diflubenzuron (Adept®, WP 25 %, Chemtura USA Corp., Middlebury, CT, USA), a chitin synthesis inhibitor; pyriproxyfen (NyGuard® EC 10, MGK®, Minneapolis, MN, USA), a juvenile hormone analogue; and azadirachtin (Azatin XL®, EC 3, OHP Inc., Mainland, PA, USA), an ecdysone agonist.

Exposure of *R. iyengari* to IGRs

To observe the effects of IGRs on endoparasitic developmental stages of mosquito larvae, we selected maximum concentration at which parasite can complete the development inside the host. Hence, the following 90 % emergence inhibition concentrations of IGRs for *Culex quinquefasciatus* (Say) were selected for this study (Mulla et al. 1986; Suman et al. 2010): pyriproxyfen 0.4 ppb, diflubenzuron 2.7 ppb, and azadirachtin 87.9 ppb. IGR impact on the nematodes was assessed by recording infectivity (percent hosts with postparasitic nematodes), parasite load (nematodes per host), sex ratio, and postparasite length and width.

The effects of IGRs on *R. iyengari* life stage were determined in three treatments. The first treatment targeted the preparasitic stage. Briefly, 100 nematodes in 30 ml of water were exposed to each IGR for 24 h. The exposure was ended by suspending the preparasitic nematodes on a 0.45-μm membrane filter followed by a 50-ml deionized water rinse. Preparasites were then transferred to 400 ml containers with 200 ml of water and 20 larvae of *C. pipiens pipiens* where infections were quickly initiated. After 5 days, mosquito larvae were transferred to individual 10 ml containers of water and examined daily for postparasitic nematode emergence. Each postparasitic nematode was removed for measurement. The second treatment examined IGR impact on the parasitic stage. Twenty mosquito larvae were exposed to 100 preparasitic nematodes in a 30-ml beaker for 3 h to

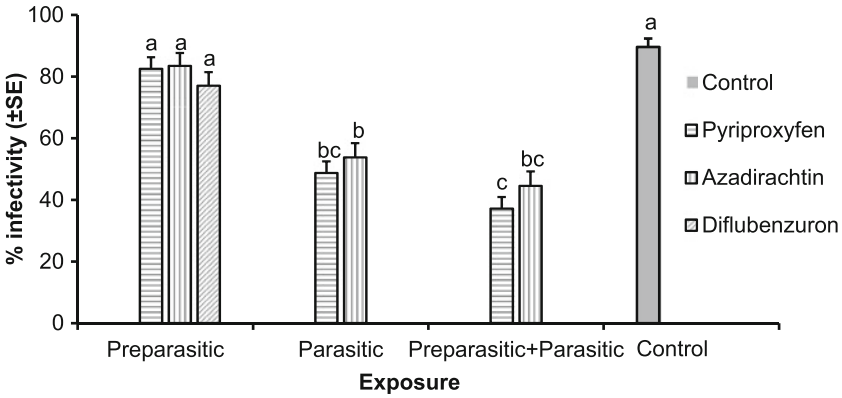

Fig. 1 The impact of insect growth regulators on infectivity of *R. iyengari* to *C. pipiens pipiens* larvae when exposed during preparasitic, parasitic, and preparasitic + parasitic stages of nematode and control. *Bars with the same letter* are not significantly different using one-way ANOVA and LSD: $F=20.32$, $df=7$, $p<0.001$

initiate host infections. The infected larvae were then treated with IGRs and monitored daily for nematode emergence as described for the first treatment. The third treatment assessed the effect of IGR exposure on both preparasitic and parasitic stages. IGR exposure was initiated at the preparasitic stage and not terminated until after 5 days of host–parasite association. The exposure was ended by rinsing larvae with deionized water on filters followed by transfer to individual 10-ml containers. Host larvae were observed daily for postparasitic nematode emergence. Controls were as described above, except that neither hosts nor nematodes were exposed to IGRs. All treatments and controls were replicated three times and the study was repeated twice. Experiments were conducted at 25±3 °C, 65±5 % RH, and 16:8 L/D photoperiod.

Statistical analysis

Unless otherwise specified, infectivity and emergence data were analyzed by one-way of analysis of variance (ANOVA), and means were separated using Fisher's least significant difference (LSD) at $p\leq0.05$. Postparasitic nematode size data (male and female length, width and length–width ratio) were measured using Image Tool ver. 3.0 freeware (University of Texas Health Science Center, San Antonio, USA) and analyzed using a nonparametric Kruskal–Wallis test at the 0.05 significance level using *H* test statistic, lower confidence limit (LCL), and upper confidence limit (UCL). Diflubenzuron data for the second and third treatments were excluded because a significant proportion of mosquito larvae died from this IGR before postparasitic nematode emergence.

Results

There were no significant differences in *R. iyengari* infectivity observed 24 h after preparasite exposure (Fig. 1). There were also no differences within parasitic and preparasitic + parasitic stages exposures between azadirachtin and pyriproxyfen treatments. However, overall infectivity was significantly lower ($p=0.001$) at parasitic and preparasitic + parasitic stages IGR exposures for azadirachtin and pyriproxyfen treatments when compared to controls.

Exposure of preparasitic nematode to the IGRs had no impact on subsequent sex ratio (Fig. 2). However, parasitic and preparasitic + parasitic stages exposure of pyriproxyfen significantly increased male emergence to 92.4±3.3 %, compared with 76.3±2.7 and 73.4±7.8 % for control and pyriproxyfen exposure of preparasitic stage, respectively ($df=7$, $f=20.3$, $p<0.001$). Conversely, increasing azadirachtin exposure did not affect nematode sex ratio.

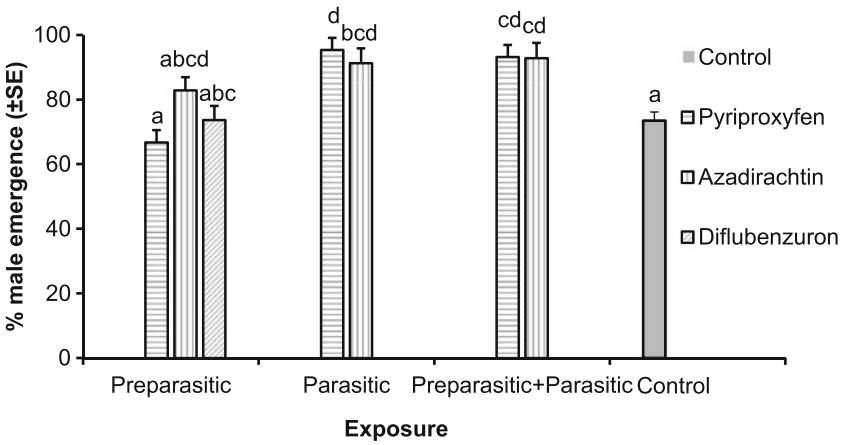

Fig. 2 The impact of insect growth regulators on average male emergence of *R. iyengari* from *C. pipiens pipiens* larvae when exposed during preparasitic, parasitic, and preparasitic + parasitic stages of nematode and control. *Bars with the same letter* are not significantly different using one-way ANOVA and LSD: $F=2.71$, $df=7$, $p=0.212$

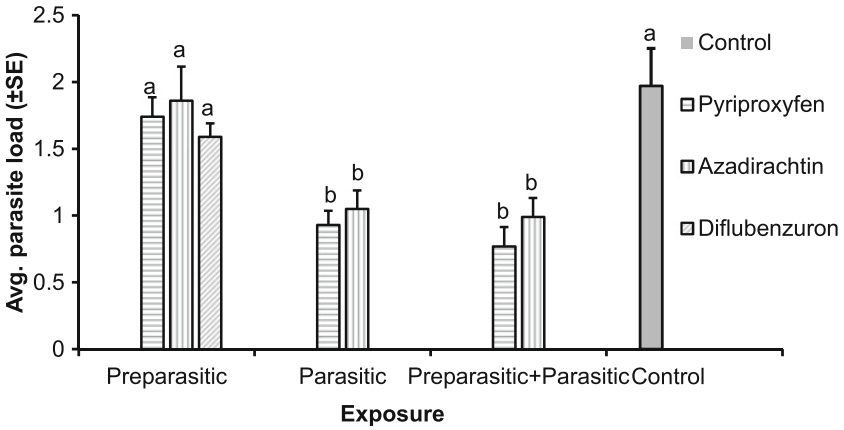

Fig. 3 The impact of insect growth regulators on average parasite load (nematodes/host) of *R. iyengari* from *C. pipiens pipiens* larvae when exposed during preparasitic, parasitic, and preparasitic + parasitic stages of nematode and control. *Bars with the same letter* are not significantly different using one-way ANOVA and LSD: $F=7.30$, $df=7$, $p=0.0001$

Parasitic load declined with increasing IGR exposure (Fig. 3). However, there was no significant difference in average parasite load observed among the IGRs and control after preparasitic exposure. Also, no difference in parasite load was observed among IGRs during parasitic and preparasitic + parasitic exposures, but when compared to preparasitic exposure, the parasite load was reduced nearly by one half, from 1.7±0.2 to 0.8±0.1 parasites/host for pyriproxyfen and from 1.8±0.3 to 1.0±0.1 parasites/host for azadirachtin ($df=7$, $f=7.30$, $p=0.0001$).

Insect growth regulators significantly impacted percent male emergence of *R. iyengari* when infections were one nematode per host for all three exposure conditions (Table 1). Preparasitic exposure to IGRs had the greatest impact on nematode male sex ratio: 9.72±5.34 to 53.34±14.23 % for control and combined IGR treatment means, respectively. Longer IGR exposures increased percent male emergence slightly, but only exposure of parasitic and preparasitic + parasitic stages to pyriproxyfen was different than preparasitic exposures to IGR (Table 1). At parasite load of 2, the impact of IGRs on percent male emergence was minimal. Although exposure of preparasitic nematodes to azadirachtin increased percent male emergence, diflubenzuron and pyriproxyfen showed virtually no effect when compared to the control. At parasitic stage exposure, both pyriproxyfen and azadirachtin treatments resulted in nearly 100 % male postparasite nematodes when parasite load was 2. At parasite load of 3, IGRs had no effect on male sex ratio.

Male nematode body length was significantly reduced at both pyriproxyfen and azadirachtin parasitic and preparasitic + parasitic stages exposures when compared to the control (H test=84.67, UCL=8.14, LCL=7, $p<0.05$) (Fig. 4). The greatest impact on male body length was observed in preparasitic + parasitic stages exposure to pyriproxyfen (6.0± 0.3 mm) when compared to unexposed nematode controls (8.6±0.2 mm). A similar trend was observed for male width except parasitic exposure to azadirachtin. No significant differences in male length–width ratios were observed within and among the IGR exposures (H test=22.83, UCL=98.18, LCL=79.45, $p<0.05$).

Female length was reduced at all IGR exposures except parasitic and preparasitic + parasitic stage exposures to

Table 1 Effects of insect growth regulators and parasite load on *R. iyengari* sex ratio

	Percent male *Romanomermis iyengari* (mean±SE)			
	Parasite load			
Exposures	1	2	3	4
Control	9.7±5.3 aA	66.5±6.5 aB	97.2±2.8 aC	100.0±0.0 aC
Preparasitic stage				
Diflubenzuron	50.9±10.9 bA	68.8±13.5 abA	100.0±0.0 aB	100.0±0.0 aB
Pyriproxyfen	50.3±14.2 bA	64.6±15.3 aA	91.2±7.3 aB	93.8±6.3 aB
Azadirachtin	60.0±14.0 bcA	87.2±6.6 bcAC	89.9±6.4 aAC	94.8±4.1 aBC
Parasitic stage				
Pyriproxyfen	84.6±8.0 cA	98.3±1.7 dB	100.0±0.0 aB	100.0±0.0 aB
Azadirachtin	66.7±12.7 bcA	100.0±0.0 dB	96.7±3.3 aB	100.0±0.0 aB
Preparasitic + parasitic stage				
Pyriproxyfen	74.7±12.7 bcA	77.1±10.4 abcAB	100.0±0.0 aB	100.0±0.0 aB
Azadirachtin	81.7±11.7 bcA	92.1±4.1 cdA	95.0±5.0 aA	100.0±0.0 aA

Means within a column followed by the same lowercase letter are not significantly different using one-way ANOVA ($p\leq0.05$). Means within a row followed by the same capital letter are not significantly different using One-way ANOVA ($p\leq0.05$)

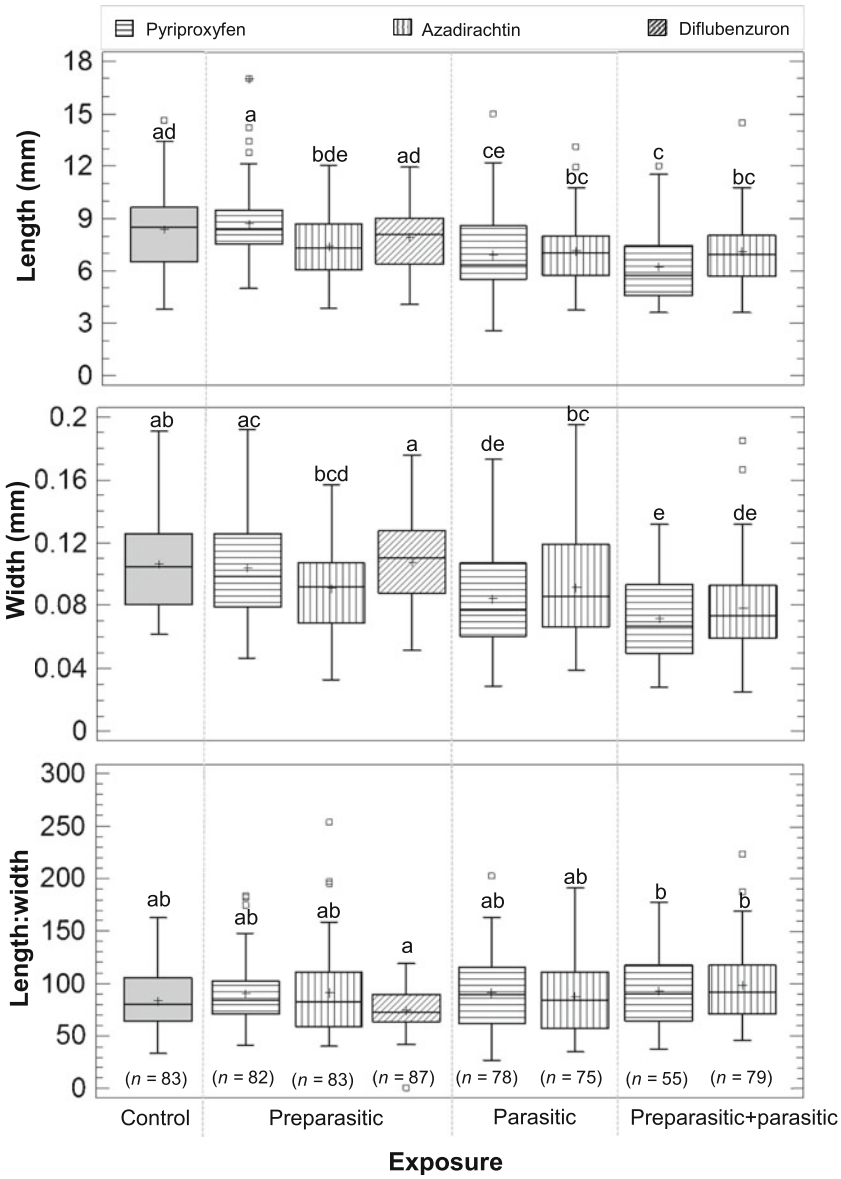

Fig. 4 Measurement of male *R. iyengari* postparasite body parameters: length, width, and length/width, following different IGR exposure conditions: preparasitic, parasitic, and preparasitic + parasitic stages exposures and control. *Horizontal black line* in the box–whisker plots is the median. *Open small box* represents outlier and *plus sign* in box plot represents mean of nematode body parameter. *Whiskers* depict confidence interval for each box plot (Kruskal–Wallis test; $p \leq 0.05$). Length: H test=84.67, UCL=8.14, LCL=7.00, df=7, $p<0.001$; width: H test=93.48, UCL=0.10, LCL=0.08, df=7, $p<0.001$; length/width: H test=22.83, UCL=98.18, LCL=79.45, df=7, $p<0.002$

azadirachtin when compared to the control (H test=18.11, UCL=14.47, LCL=11.21, $p<0.05$) (Fig. 5). The IGR treatments did not impact female width or female length–width ratio.

Discussion

Our results show little compatibility between *R. iyengari* and IGRs. Increasing pyriproxyfen and azadirachtin exposure duration decreased infectivity, parasite load, and male and female body length and skewed sex ratio heavily toward male production. This is the first report on length reduction of female postparasitic nematodes and disruption of sex ratio at lower parasite load as a result of preparasitic, parasitic, and preparasitic + parasitic stages exposures to IGRs. Preparasitic nematodes exposed to IGRs for 24 h showed no significant impact on parasite load, infectivity, sex ratio, and male size. In contrast, preparasitic exposure to diflubenzuron and pyriproxyfen reduced female length while male nematode percent emergence was increased at a parasite load of one for all three IGRs. Diflubenzuron is best explained by the mode of action of the IGR as a chitin synthesis inhibitor and that *N*-acetylglucosamine a related chitin polymer is present in preparasites (Kerwin et al. 1990). Dennis (1976) showed that final molt from larva to adult of the nematode *Nematospiroides dubius* (Baylis) was stimulated or inhibited by α-ecdysone and synthetic juvenile insect hormones, respectively. However, Levy and Miller (1977) and Finney et al. (1977) showed that the infectivity and viability of *R. culicivorax* were not affected by diflubenzuron with 24 h exposure.

Development of parasitic stage of *R. culicivorax* depends on the nutrition from host tissues and results in host weight

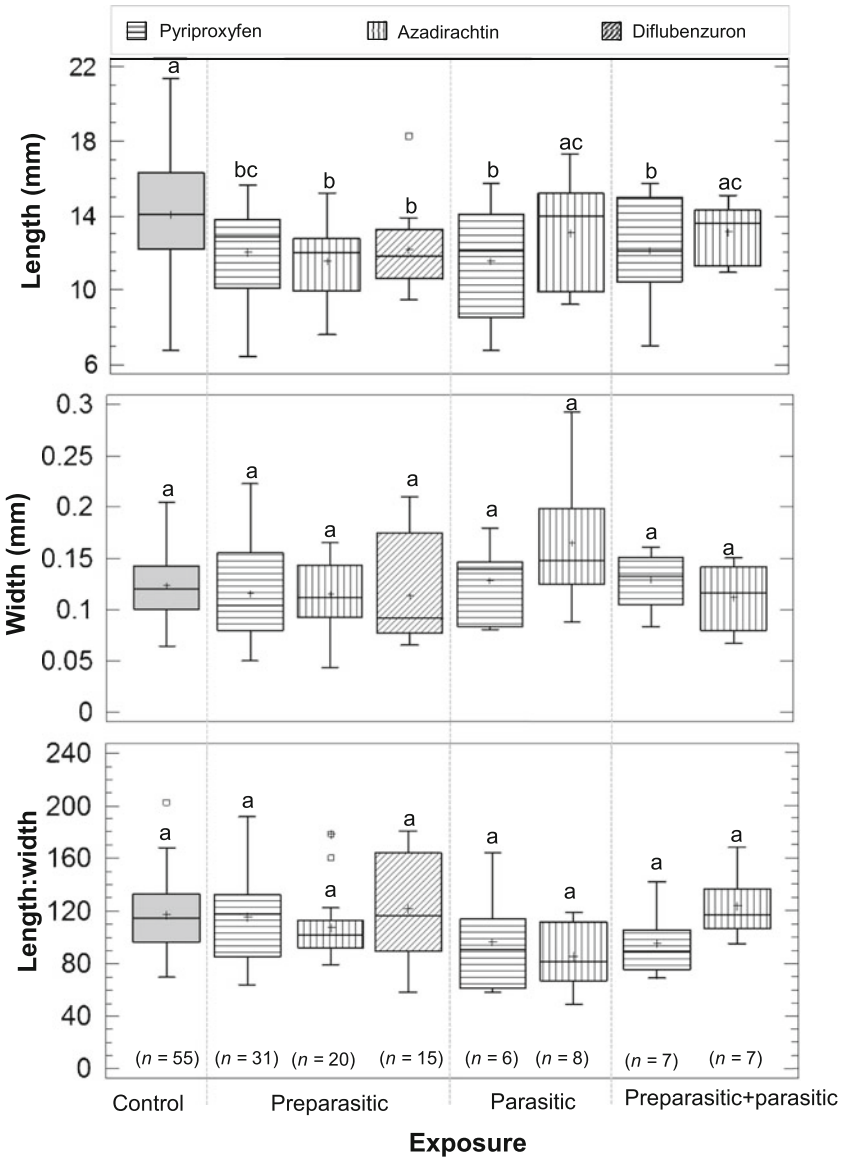

Fig. 5 Measurement of female *R. iyengari* postparasite body parameters: length, width, and length/width, following different IGR exposure conditions: preparasitic, parasitic, and preparasitic + parasitic stages and control. *Horizontal black line* in the box–whisker plots is the median. *Open small box* represents outlier and *plus sign* in box plot represents mean of nematode body parameter. *Whiskers* depict confidence interval for each box plot (Kruskal–Wallis test; $p \leq 0.05$). Length: H test=18.11, UCL=14.47, LCL=11.21, df=7, $p<0.01$; width: H test=10.22, UCL=0.15, LCL=0.10, df=7, $p<0.18$; length/width: H test=14.31, UCL=130.88, LCL=94.12, df=7, $p<0.05$

loss (Giblin and Platzer 1985). Gordon et al. (1981) found that a low protein diet fed to *R. culicivorax*-parasitized *Aedes aegypti* resulted in asynchronous parasitic development and the emergence of incompletely developed postparasites. Reduced postparasite survival was noted when the nutritionally deprived hosts were infected by more than one parasite.

The average parasite load was more than one per host in both control and treatment exposures indicating normal nematode development when IGRs were absent. When nematodes developing inside the host were exposed, however, several traits were negatively affected, including infectivity, sex ratio, and body size. In contrast, parasite and preparasitic + parasitic stages exposures to IGR reduced nematode development as indicated by parasite load of ≤1. This finding suggests that IGR-intoxicated larvae are less able to support a high parasite load and therefore adversely affect parasite fitness. We attribute this to insufficient mosquito nutrition caused by IGRs. Azadirachtin, fenoxycarb, and JHAs reduce food intake, causing poor growth of insect larvae (Leonardi et al. 1996; Kumar and Parmar 1998).

The sex ratio of emerged *R. iyengari* shifted from 90 % female to strongly male biased (50–84 %) when exposed to pyriproxyfen or azadirachtin at a parasite load of one. At parasite loads of 2, male sex ratio was enhanced by azadirachtin in all the three exposures and by pyriproxyfen in parasitic nematode exposure when compared to control. In contrast, at higher parasite load, IGRs had no impact on nematode sex determination. Our study was not a direct test of the environmental sex determination (i.e., host quality) model as described by Charnov and Bull (1977). Environmental sex determination is favored by natural selection when an individual's fitness as a male or female is strongly influenced by environmental conditions and where the individual

has little control over which environment it will experience (Charnov and Bull 1977). This predicts greater reproductive success (i.e., fitness) for female parasites when environmental conditions are optimal, and a predominance of males when growth conditions are poor. Parallel outcomes can be drawn, however, because environmental sex determination occurs in all mermithid species (Petersen 1972). Similarly, Petersen (1977) found as nematode density increased a greater proportion of males emerged from the mosquito host. Although in our study male bias was observed with hosts with two nematodes, Petersen (1977) did not observe bias until nematode density reached ten parasites/host. This is not surprising since the influence of superparasitism before the production of males is equal to females varies widely in the family Mermithidae (Korpelainen 1990). Petersen (1972, 1977) and Gordon et al. (1981) showed that host size, nutrition level, and parasite load affect mermithid sex determination. All of these, however, can be related back to host nutrition.

Exposure of insect growth regulators to *R. iyengari* following preparasitic, parasitic, and preparasitic + parasitic stages treatments resulted in a decrease in body length of postparasitic nematodes. However, in our study, the length to width body ratio for both genders was unaffected, suggesting that the nematodes maintained their body shape under unfavorable conditions even when their body length was reduced. Variation in body size is a common feature among helminth and is a predictor of parasite reproductive potential (Poulin 1998). Temporal and spatial competition among nematodes developing within the same host can result in disparate adult body size (Poulin and Latham 2002). Tingley and Anderson (1986) also revealed that reduced reproductive potential of mermithid females related to the reduction in *R. culicivorax* body size. Paily and Balaraman (1994) showed that male and female body length of *R. iyengari* decreased with increased temperature. For population dynamics, the contributions of large female nematodes were found to be more in buildup of the population due to high fecundity (Dobson 1986). We observed that IGR exposures skewed the nematode population towards small size, which would likely dampen population growth. Therefore, both a decrease in nematode length and female production could lead to decreased fitness of a mermithid population and unfavorable changes in population dynamics.

In conclusion, pyriproxyfen, azadirachtin, and diflubenzuron were incompatible with *R. iyengari* and affects infectivity, parasitic load, sex ratio, and body size of the nematode. These factors may destabilize the dynamic of host–parasite population interactions and affects the regulatory role of parasites (May and Anderson 1978). Our data indicated that host nutrition has an indirect negative relationship on several important biological factors of *R. iyengari*. The use of these IGRs in locations where mermithid populations are naturally present should be cautioned.

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