Effect of treatment using *Carica papaya* seed extract with Ag–TiO$_2$ nanocomposite on the mortality of *Aedes aegypti* larvae

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Abstract. The incidence and mortality rate of dengue hemorrhagic fever (DHF) in Indonesia have continued to increase. However, the insecticidal control of the vector of DHF, *Aedes aegypti* (yellow fever mosquito), has resulted the development of insecticide resistance. The aim of this study was to evaluate the effects, singly and in combination, of two components with proven larvicidal activity, namely, the Ag–TiO$_2$ nanocomposite and *Carica papaya* seed extract, on the mortality of *A. aegypti* larvae. This experimental study was categorized into the control and three intervention groups: 1) papaya seed extract at concentrations of 0, 2, 4, 6, 8, and 10 ppm; 2) Ag–TiO$_2$ at concentrations of 0, 5, 10, 15, 20, and 25 ppm; and 3) mixture of Ag–TiO$_2$ and *C. papaya* seed extract 50% and 90% lethal concentration. After 24 h exposure, the 50% lethal concentration and the 90% lethal concentration were determined for the Ag–TiO$_2$ nanocomposite (5.19 ppm and 10.87 ppm, respectively) and the *C. papaya* seed extract (25.98 ppm and 44.30 ppm, respectively). Significant differences were detected among Ag–TiO$_2$ concentrations and mixture-group concentrations ($p < 0.05$). Significant positive correlations were detected between larval mortality and Ag–TiO$_2$ concentrations and mixture group concentrations ($r = 0.812$, $p = 0.001$ and $r = 0.343$, $p < 0.001$, respectively). Changes in the visual appearance of dead larvae following treatment with nanocomposites and/or seed extract included damage to the abdominal segments, increased transparency of the abdomen, and reduced brush numbers. Therefore, the addition of the nanocomposite Ag–TiO$_2$ to *C. papaya* seed extract increased the larvicidal effectiveness of the seed extract against *A. aegypti* larvae.

1. Introduction

Dengue hemorrhagic fever (DHF) is a dengue virus infection, transmitted by the *Aedes aegypti* mosquito vector, which has become an endemic disease, affecting 128 tropical and subtropical countries [1,2].
Southeast Asia and the Western Pacific fall within the DHF endemic region because approximately 70% of the population at risk of DHF live in this area [3]. By 2016, the spread of DHF in Indonesia had reached 85% districts/cities infested with *A. aegypti* [4]. One reason behind the increase in DHF is the climatic changes, which have increased the proliferation of the mosquito vector [4,5]. To date, vector control using environmental, biological, and chemical approaches has been the focus of reducing the incidence of DHF. Chemical control, using synthetic insecticides, such as organophosphates and pyrethroids, has resulted in the evolution of insecticide resistance in *A. aegypti* mosquitoes [6,7].

As alternatives to synthetic insecticides, phytochemicals of plants, such as saponins, alkaloids, terpenoids, and flavonoids, have been shown to kill *A. aegypti* larvae, and should be able to control insecticide-resistant mosquitoes. One plant tissue rich in such phytochemicals is the *Carica papaya* seed. Several studies have demonstrated that *C. papaya* seeds have antimicrobial [8,9], antioxidant [8] and antiprotozoal [10] activities and chemopreventative effects against certain cancers [11]. Malathi and Vasugi [12] reported that an ethanol extract of the seeds of *C. papaya* exhibited strong larvicidal activity against *A. aegypti* larvae compared to extracts of the other fruit components, such as skin and flesh. Exposure for 24 h to an ethanol extract of *C. papaya* seeds resulted in 100% mortality of fourth instar *A. aegypti* larvae [12], with an LC50 of 0.48 mg/mL [13]. Biologically active phytochemicals contained in *C. papaya* seeds include flavonoids, tannins, and alkaloids. Tannins can inhibit the synthesis of cell proteins by forming irreversible complexes with proline-rich proteins [14], whereas alkaloids affect protein kinases, disrupting signal transduction and tissue developmental processes [15].

To prevent the development of resistance in the vector and to increase the effectiveness of larvicidal activity, the use of combinations of larvicidal agents with different modes of action are being explored [16]. Silver (Ag) nanoparticles and titanium dioxide (TiO2) have been shown to exhibit useful larvicidal [17,18], antifungal [19], and antibacterial activities. Studies by Dhanaleksmi et al. [9] showed that TiO2 and silica, which act as the surface cover of Ag nanoparticles, increase the antibacterial activity of Ag nanoparticles. In addition, TiO2 nanoparticles can cause cell death by increasing the formation of reactive oxygen species [20-22], whereas Ag nanoparticles achieve antifungal effects by inactivating cell sulfydryl groups, resulting in cell death [19]. Production of the nanocomposite Ag–TiO2 requires an ultraviolet light source; UV rays can be used in these studies because they do not affect the survival of larvae [23].

In this study, the hypothesis that combining the Ag–TiO2 nanocomposites with *C. papaya* seed extracts could increase the larvicidal activity against *A. aegypti* was tested. The 90% (LC90) and 50% (LC50) lethal concentrations were determined for *C. papaya* seed extract (2–10 ppm) alone and in combination with the nanocomposite Ag–TiO2 (5–25 ppm). In addition, we also assessed the morphological changes in *A. aegypti* larvae exposed to different treatments.

2. Methods

2.1 Preparation of *C. papaya* seed extract

Seeds were separated from *C. papaya* fruit purchased from local markets. They were washed, and dried. Then, the dried seeds were cut into fine pieces and allowed to dry at room temperature for 2 weeks. The dried seeds were ground to produce a fine seed powder. A sample (125 g) of the seed powder was extracted by suspension in 100 ml 70% (v/v) ethanol for 24 h on an orbital shaker. After the extraction process was completed, the suspension was filtered and the filtrate stored. The ethanol filtrate was evaporated to dryness using a vacuum evaporator, and the extract was weighed. The dried seed extract (5.3 g dry weight) was dissolved in 106 mL distilled water to produce a 5% (w/v) stock solution (crude extract).

2.2 Preparation of nanocomposites

This work was carried out in the Department of Chemical Engineering, Faculty of Engineering, Universitas Indonesia. The Ag–TiO2 nanocomposite was formed using photo-assisted deposition (PAD) method. First, up to seven drops of concentrated nitric acid were added to 650 mL 10% (v/v) aqueous methanol to lower the pH to 3. To the solution were added 1.98 g Degussa P25 TiO2, which was stirred
for 5 min, after which the solution was subjected to sonication for 30 min. Then, 0.0314 g of 1% AgNO₃ was added. The mixture was irradiated with constant stirring in a container around which four 10 W UV lamps were mounted for 6 h. After UV treatment, the resulting solution was cloudy, and hence, was clarified by centrifugation. The solution was then heated on a hotplate stirrer at 150°C until dry. The resulting powder was ground with a mortar and pestle, after which calcination was carried out in a furnace at 300°C for 2 h, resulting in a yield of 2 g dry weight of grayish-purple nanocomposites. A sample (0.5 g) of Ag–TiO₂ nanoparticles were suspended in 100 mL distilled water to produce a stock solution of 0.5% (w/v) nanoparticles. Because the nanoparticles settled out of suspension very rapidly, the stock solution was vortexed thoroughly before aliquots were taken.

2.3 Preparation of group treatment solutions

Treatment group I contained dilution series of the 5% (w/v) C. papaya crude extract stock solution to produce concentrations of 2, 4, 6, 8, and 10 ppm in distilled water. The treatment group II contained 0.5% (w/v) Ag–TiO₂ nanocomposites with concentrations of 5, 10, 15, 20, and 25 ppm in distilled water. The concentration ranges were selected based on other published studies. The third treatment group, III, contained a mixture of 15 ppm C. papaya seed extract and nanocomposite. There were no treatment for control group.

2.4 Treatment of larvae with extracts and/or nanocomposites

Treatment groups containing larvae and preparations of extracts and/or nanocomposites were observed after 6 h, 24 h, and 48 h incubation.

2.5 Observation of larval morphology

Dead larvae were randomly selected from each treatment group and from each concentration of the treatment group. Morphological changes to the head, abdominal, chiffon feather, and anal segments were observed using a light microscope. To observe tracheal changes in spiracles and papillae, the electron microscopy facilities at the Parasitological Laboratory of the Faculty of Medicine, University of Indonesia were used.

2.6 Data analysis

Data were analyzed using SPSS version 20. First, data distribution of variables was tested for normality using the Shapiro–Wilk test, with data from two treatment groups and one control group. If the data distribution was normal, data analysis was carried out with the parametric one-way ANOVA test. However, if the data distribution deviated significantly from normality, then data analysis was carried out with the non-parametric Kruskal–Wallis test. In both cases, appropriate Post-Hoc tests were used to carry out multiple pairwise comparisons. To determine the LC₉₀ and LC₅₀, a regression analysis of the relationship between concentration and mortality was carried out.

3. Results

The collected C. papaya seeds had a fresh weight of 3500 g, whereas, after drying, the C. papaya seed powder had a dry weight of 500 g. A sample (125 g) of the seed powder was extracted with 70% ethanol, to produce 5.3 g dry weight seed extract. The Ag–TiO₂ nanocomposite used in this study was formed by combining Ag nanoparticles obtained from AgNO₃ and TiO₂ nanoparticles. Table 1 shows the number of dead larvae observed, as well as the LC₉₀ and LC₅₀ calculated at 24 h and 48 h in various treatment groups of the C. papaya seed extract. Normality test results showed the percentage of larval mortality at 24 h to being normally distributed (p > 0.05). As a consequence, ANOVA tests were performed on data from the C. papaya extract group and the Ag–TiO₂ nanocomposite group. However, in the mixture group, data were not normally distributed; therefore, analysis was carried out by the Kruskal–Wallis test.

ANOVA of the effect of the seed crude extract concentration on larval mortality showed no significant difference (p = 0.198) at 24 h, which means that the concentration of the crude extract did not affect larval mortality at that time. However, at 48 h, ANOVA showed significant differences between larval mortality at the various extract concentrations. This shows that the concentration of C. papaya extracts affected the
survival of larvae at 48 h. In contrast to the extract treatment group, ANOVA of the Ag–TiO₂ nanocomposite concentrations (p < 0.001) and the Kruskal–Wallis test of both mixed groups (p < 0.001) showed significant differences. This means that the Ag–TiO₂ nanocomposites and mixture both exhibited a dose-dependent effect on the mortality of *A. aegypti* larvae.

**Table 1.** Effect of *C. papaya* seed extract on *A. aegypti* larval deaths at 24 h and 48 h exposure on 100 larvae.

| Concentrations of *C. papaya* seed extract (ppm) | Dead larvae at 24 h and 48 h | Lethal concentrations at 24 h and 48 h |
|--------------------------------------------------|------------------------------|---------------------------------------|
|                                                  | 24 h mean ± SD               | 48 h mean ± SD                        | LC₅₀ | LC₉₀ | LC₅₀ | LC₉₀ |
| 0                                                | 0                            | 0                                     | 0.0  | 0.0  | 0.0  | 0.0  |
| 2                                                | 1.25 ± 1.26                  | 1.75 ± 0.96                           | 25.98| 44.30| 8.37 | 16.52|
| 4                                                | 2.5 ± 2.52                   | 11 ± 5.35                             |      |      |      |      |
| 6                                                | 2.5 ± 2.65                   | 13.25 ± 4.35                          |      |      |      |      |
| 8                                                | 3.25 ± 1.89                  | 12 ± 3.92                             |      |      |      |      |
| 10                                               | 2 ± 0.82                     | 10.75 ± 6.08                          |      |      |      |      |

SD: Standard deviation
LC₅₀: Lethal concentration 50%
LC₉₀: Lethal concentration 90%

Table 2 shows the number of dead larvae at the 6, 7, 24, and 48 h exposure to nanoparticles in the Ag–TiO₂ group. In contrast to the extract group, the Ag–TiO₂ nanocomposites showed 100% mortality of larvae at each concentration after 48 h, preventing the use of ANOVA. Table 2 also shows that, at 7 h, 50% larval death was already observed at concentrations of 15 and 20 ppm. The lowest concentration (5 ppm) reached the 50% larval mortality by 24 h. Compared with Table 1, the LC₅₀ and LC₉₀ values after 24 h and 48 h exposure to the treatment groups of nanocomposites Ag–TiO₂ were markedly lower than the corresponding values in the extract group. This finding shows that the nanocomposites are more toxic than the *C. papaya* extracts, with the LC₅₀ and LC₉₀ values for the extract being 5.19 and 10.87 ppm, respectively, compared to 2.55 and 3.41, respectively for the nanocomposites.

**Table 2.** Effect of Ag–TiO₂ nanocomposites on *A. aegypti* larval mortality at 24 h and 48 h on 100 larvae.

| Nanocomposite Ag–TiO₂ Concentration (ppm) | 6 h Dead larvae (Mean ± SD) | 12 h Dead larvae (Mean ± SD) | 24 h Dead larvae (Mean ± SD) | 48 h Dead larvae (Mean ± SD) |
|-------------------------------------------|-----------------------------|------------------------------|-------------------------------|-----------------------------|
| 0                                         | 0                           | 0                            | 0                             | 0                           |
| 5                                         | 0                           | 0                            | 13.25 ± 0.96                  | 25 ± 0                      |
| 10                                        | 0.75 ± 0.96                 | 1 ± 0.82                     | 25 ± 0                        | 25 ± 0                      |
| 15                                        | 4 ± 0.82                    | 12 ± 0                       | 25 ± 0                        | 25 ± 0                      |
| 20                                        | 6 ± 0.82                    | 19 (18–20)*                  | 25 ± 0                        | 25 ± 0                      |
| 25                                        | 5 (4–5)*                    | 5 ± 0.82                     | 24.25 ± 0.96                  | 25 ± 0                      |

* Median (minimum–maximum)
SD: Standard deviation

Table 3 shows the number of dead larvae in the treatment group and the mixture of both nanocomposites and extract after 6 h, 7 h, 12 h, 24 h, and 48 h exposure of 100 larvae. At 12 h, the mortality of the larvae
reached 50% at extract concentrations of 2 and 4 ppm, while, at the highest concentration (10 ppm), larval mortality at 24 h reached 50%.

**Table 3.** Effect of mixed *C. papaya* extract and nanocomposite Ag–TiO₂ on mortality of *A. aegypti* larvae after 24 h and 48 h on 100 larvae

| Concentrations (ppm) | Dead larvae at (h) |
|----------------------|---------------------|
|                      | 6 h Mean ± SD       | 12 h Mean ± SD   | 24 h Mean ± SD   | 48 h Mean ± SD   |
| 0                    | 0                   | 0                 | 0                 | 0                 |
| 2 + 15               | 4.5 (4–5)*          | 13 (12–13)*       | 25 ± 0            | 25 ± 0            |
| 4 + 15               | 0                   | 17.25 ± 0.96      | 24 (23–24)*       | 25 ± 0            |
| 6 + 15               | 2 ± 0               | 8 (7–8)*          | 25 ± 0            | 25 ± 0            |
| 8 + 15               | 1 ± 0               | 11.25 ± 0.96      | 24 (24–25)*       | 25 ± 0            |
| 10 + 15              | 1 (0–1)*            | 2.5 (2–3)*        | 13.25 ± 0.96      | 25 ± 0            |

* Median (minimum–maximum)
SD: Standard deviation

**LC₅₀** values in mixed ingredients groups after 24 h showed negative results of −1.48. However, the **LC₉₀** of the mixed treatment group of the extract + nanocomposite group (10.67) was lower than that of the nanocomposite group Ag–TiO₂ (10.87) and the *C. papaya* extract group. This finding shows that the Ag–TiO₂ nanocomposites were not only toxic but also increased the toxicity of the seed extract for the *A. aegypti* larvae although the increased toxicity of the mixture treatment group was not significantly different from that of the nanocomposite Ag–TiO₂ treatment group. However, after 48 h, the **LC₅₀** and **LC₉₀** values of the mixture treatment groups (1.02 and 1.40 ppm, respectively) were lower than the corresponding values for Ag–TiO₂ nanocomposites (2.55 and 3.41 ppm, respectively) and *C. papaya* extract.

Table 4 shows the comparison of larval mortality in the Ag–TiO₂ nanocomposites treatment group and the both mixture treatment groups with respect to the mortality of *A. aegypti* larvae after 6 h, 12 h, 24 h, and 48 h on 100 larvae. In both mixed groups there was a decrease in larval mortality after 6 h, 12 h, and 24 h exposure, compared to larval mortality in the nanocomposite group. This suggests that the effect of the Ag–TiO₂ nanocomposites is inhibited when it is added with *C. papaya* extract.

**Table 4.** Comparison of larvae deaths in the nanocomposite Ag–TiO₂ treatment group and the second mixture on larval mortality at 6 h, 12 h, 24 h and 48 h

| Treatment                          | Dead larvae at (h) |
|------------------------------------|---------------------|
|                                    | 6 h Mean ± SD       | 12 h Mean ± SD   | 24 h Mean ± SD   | 48 h Mean ± SD   |
| Nanocomposite (15 ppm)             | 4 ± 0.82            | 23.75 ± 1.26     | 25 ± 0            | 25 ± 0            |
| Nanocomposite (15 ppm +) *C. papaya Extract (4 ppm) | 0                   | 17.25 ± 0.96     | 24 (23–24)*       | 25 ± 0            |

*Median (minimum–maximum)
SD: Standard deviation

Subsequently, linear correlation analyses were carried out to quantify the relationships between concentration and larval mortality, and significant positive correlations were obtained for the *C. papaya* extract.
extract group \( (r = 0.418, p = 0.241) \), Ag–TiO\(_2\) nanocomposite \( (r = 0.812, p < 0.001) \), and the mixture group \( (r = 0.343, p < 0.001) \) after 24 h. In addition, linear regression analyses quantified the concentration/mortality relationship for each treatment group. The relationship formula of extract concentration of \( C. \) \textit{papaya} \((x)\) with percentage of \( A. \) \textit{aegypti} larvae mortality \((y)\) was \( y = 0.229x + 0.774 \) (non-significant) with confidence interval 95\% \(-0.557–2.105\). The corresponding formulae for the nanocomposites was \( y = 0.894x + 7.571 \) with 95\% confidence interval 3.269–11.874 (significant), whereas that for the mixture was \( y = 0.932x + 13.881 \) with confidence interval 95\% 7.054–20.708 (significant).

![Morphology of dead A. aegypti larvae following exposure to: (a) extract of C. papaya seed, (b) Ag–TiO\(_2\) nanocomposite and (c) mixture of C. papaya and Ag–TiO\(_2\) nanocomposite extracts](image)

**Figure 1.** Morphology of dead \( A. \) \textit{aegypti} larvae following exposure to: (a) extract of \( C. \) \textit{papaya} seed, (b) Ag–TiO\(_2\) nanocomposite and (c) mixture of \( C. \) \textit{papaya} and Ag–TiO\(_2\) nanocomposite extracts

Morphological changes occurring in typical dead larvae following exposure to the ethanol extract of \( C. \) \textit{papaya} seed, Ag–TiO\(_2\) nanocomposites, or to a mixture of both, showed similarities, such as changes in the color of the abdomen, which became more transparent, and damage to the abdominal segment (Figure 1). However, there was a difference between larvae exposed to Ag–TiO\(_2\) nanocomposites with those exposed to \( C. \) \textit{papaya} seed extract and a mixture of both; there was a reduction in the number of brushes.

### 4. Discussion

The increasing incidence of mortality due to DHF can be resolved by breaking the reproduction chain of the dengue virus i.e. by controlling the reproduction of the vector \( A. \) \textit{aegypti}. The increase in DHF cases is also influenced by an increase in the number of DHF vectors [5]. Until now, the method used to control the vector has been the use of chemical pesticides, such as pyrethroids and organophosphates. However, the use of chemical pesticides over long periods of time can lead to resistance in the insect [4]. In Indonesia, the frequency of resistance to organophosphate group larvicides in Bantul, Yogyakarta was low (2.22\%) [24], but the problem will increase over time. Therefore, a complex larvicide such as \( C. \) \textit{papaya} seed extracts is required whereby the likely presence of multiple active components will greatly delay the evolution of resistance. Furthermore, to enhance the effectiveness of such larvicides, Ag–TiO\(_2\) nanocomposites [25,26], which have been shown to exhibit larvicidal properties [17,18], probably via a different mode-of-action to \( C. \) \textit{papaya} seed extract, could increase the activity and longevity of the effect of the seed extract on mosquito larvae.

Compared to the research reported by Chokkun, the current study used the same solvent, 70\% ethanol, but did not use 0.1\% (v/v) DMSO [13]. The use of DMSO had a complicating effect on the response of
the larvae to the extract, with the LC50 of the C. papaya seed extract used in the current research (25.98 ppm) being higher than that reported by Chokkun (4.8 ppm) [13]. The use of Ag-TiO2 nanocomposites as an anti-bacterial agent has been widely researched [17,18,25,26], but research into its larvicidal properties has not been reported. In this research, the used nanocomposite Ag–TiO2 was formed by using PAD method, which was also use by the Afrozi research group [27]. Morphological changes that occurred in each treatment group included a change towards greater transparency of the abdomen and damage to the abdomen. These findings are in agreement with the research reported by Saranya et al. who identified damage to the digestive tract and dechitinized larvae in A. aegypti larvae exposed to leaf extracts [28].

4.1 Correlation between C. papaya seed extract dosage and larval mortality
At 24 h, ANOVA did not detect any significant difference in larval mortality between the six different concentrations of C. papaya seed extract. However, at 48 h, significant differences were detected by ANOVA. This may be because the active ingredients in the seed extract were at low concentrations; therefore, it took 48 h before an effect was observed. This relates to the mode-of-action of C. papaya seed extract as a larvicide, as described in the Shimada study and the Goel et al. research, where tannins in extracts inhibited protein function, whereas alkaloids inhibited the process of cell and tissue development and affected protein kinases in signal transduction processes [14,15]. Based on the research of Malathi et al. [12], using ethanol as the solvent for extraction of papaya seed powder, the active ingredients of alkaloids, flavonoids, tannins, anthocyanins, and coumarins were at a higher concentration in the ethanol extract than in an aqueous extract of the same seed powder. In addition, research by Hayatie et al. [29] showed that tannins and alkaloids in C. papaya seed extracts were at a higher concentration than in the papaya skin. In addition to the alkaloid and tannin contents, C. papaya seeds also contain high concentrations of oleic and linoleic acids. Rahuman et al. stated that oleic and linoleic acids isolated from Citrullus colocynthis, using petroleum ether as the solvent, exhibited potent larvicidal activity, with LC50 values of 8.80 and 18.20 ppm, respectively, at 24 h [30]. These findings show that the content of oleic acid and linoleic acid, which are present in C. papaya seeds, are more efficiently extracted using petroleum ether solvent in order to achieve the most potent larvicidal activity.

4.2 Correlation of nanocomposite Ag–TiO2 with larval mortality
At 24 h, some nanocomposite concentrations (10, 15, and 20 ppm) caused 100% mortality of the larvae. This was probably due to the mode-of-action of Ag–TiO2 nanocomposites, causing cell lysis, due to Ag nanoparticles, and cell apoptosis due to TiO2 nanoparticles [18,19]. The study by Velmurugan et al. investigating the antifungal effect of Ag nanoparticles, reported that these nanoparticles caused cell lysis by disrupting membrane energy metabolism, causing fungal DNA mutations, and separating the enzyme complexes necessary for respiratory and membrane regeneration [19]. The study by Murugan et al. that used TiO2 nanoparticles as larvicides, stated that TiO2 caused cell apoptosis by degrading protein, lipid, and DNA, causing organellar and cell death [18]. The current study is the first report of Ag–TiO2 nanocomposites having larvicidal effects, with the LC50 and LC90 values of Ag–TiO2 at 24 h being 5.19 ppm and 10.87 ppm, respectively [18].

4.3 Interaction between seed extract and Ag–TiO2 nanocomposites with respect to larval mortality
At 24 h, the mixed group of Ag–TiO2 nanocomposite and C. papaya seed extract showed LC50 and LC90 values of −1.48 and 10.67, respectively. The negative LC50 indicates that the solution is promoting growth probably because of the interactions between the Ag–TiO2 nanocomposite and C. papaya seed extract, with C. papaya seed extracts causing turbidity in the treatment container and blocking the ultraviolet light required by the Ag–TiO2 nanocomposites to form. This inhibits the performance of the nanocomposites; hence, it was not until 48 h that 100% mortality is observed. In addition, the material of the plastic container may also inhibit the incoming ultraviolet rays, delaying the formation of nanocomposites.
5. Conclusion
It was concluded that the addition of Ag-TiO2 to C. papaya seed extract further increased the larvicidal effect of this seed extract against A. aegypti larvae. The larvicidal effects were also associated with visible morphological changes, such as damage to the abdominal segment and greater transparency of the abdomen.

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