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Efficacy of pepper leaf powder (*Piper nigrum*) in the sex redirection of tilapia (*Oreochromis niloticus*)

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Abstract
This study focused mainly on determining the concentration that will produce the highest male percentage using the Pepper Leaf Powder (PLP). It also evaluated the effect of PLP on the growth and survival of the treated fish and the cost analysis on the use of the indigenous hormone. The study was done into two phases: Range Finding Test and Definitive Test Run (DTR). For DTR, the PLP inclusion levels were tested at 0.5% interval per treatment. The treatments were: T_1 with 5.5% PLP, T_2 with 6.0% PLP and T_3 with 6.5% PLP inclusion. Newly hatched fry were stocked in 50-L aquarium and fed *ad libitum* with the PLP-treated feeds for 28 days. Then the fish were nursed in tank for 35 days after which the sex of each fish was determined by examination of the genital papilla. The samples that sex was not easily identified were subjected to gonadal examination. Mean percentage male ranged from 72% (T1) to 80% (T3). However, statistical analysis for the percentage male for the DTR showed no significant differences among the treatment means. Increasing the inclusion of PLP in the feed also increases the percentage of male which indicates that higher concentration of PLP should be tried in future studies to increase the percentage sex reversal. Treatment 3 showed the highest value in terms of weight gain (15.64g ± 0.46), length gain (91.55 mm ± 1.82). and survival rate (84.8%). In the cost and return analysis, net profit of PhP 2,706.8 with Php 1.1 per fish and ROI of 38.92% were attained using PLP in the sex redirection of tilapia.

Keywords: ad libitumly, indigenous hormone, sex redirection

Introduction

In the recent years, tilapia is considered as one of the best global aquaculture candidates and the second most important farmed fish in the Philippines next to milkfish (*Chanos chanos*). Their characteristics such as rapid growth, high tolerance to low water quality, resistance to disease, and ease of spawning, and good consumer acceptance. These factors make them suitable fish for ponds, cages, and pens. However, one problem with this fish is their early maturation and ability to breed every month being a prolific species. This condition results in the overpopulation of stocked tilapia and the stunting of growth because of the crowding of the fish (Fashina-Bombata and Megbowon, 2012) [5]. Another problem is the sizes of the fish at harvest, varying from small to large due to the faster growth of males. This also makes it more difficult to establish uniformity of harvested stocks. For producers wanting high yields of largesized fish in 6 months, they prefer all-male fry.

Sex reversal or sex redirection is the process of giving exogenous steroids during the gonadal development period that can control the phenotype overriding the expression of the genotypically determined sex (Phelps and Popma, 2000) [15]. It is one of the various techniques being developed to control unwanted reproduction of the stocked fish. Tilapia at an early stage of development is sexually undifferentiated. Methyltestosterone is the most commonly used androgen to direct the sex of tilapia and is considered to be the most simple and reliable way to produce all-male tilapia stocks. The production of all-male fry through administration of androgen such as 17α-methyltestosterone is also considered to be the most effective and economically feasible method for obtaining all-male tilapia populations.

There is however a global concern about the effect of this steroid on the fish flesh, consumers (man), and the environment (water bodies into which effluent is released). People demand and consume organic products because they believe that these products are safer and healthier for both human beings and the environment. For instance, restrictions exist in some countries regarding the sale of hormone-treated fish unless it is proven that there are no risks to human health from consumption. It was reported that the marketing of treated fish is illegal in EU countries and India (White *et al.*, 2006) [21].

As a result, fish farmers demanded some alternatives. In a study by Kavitha and Subramanian (2011) [11], they suggested the use of natural substances while addressing the concerns about hormone-treated fish. Animal hormones and plant hormones can also be used as an organic stimulants. Plant extracts containing diverse bioactive principles such as alkaloids, flavonoids, pigments, phenolics, terpenoids, steroids, and essential oils have been reported to promote various activities like antistress, growth promotion, appetite stimulation, tonic and immunostimulation, and antimicrobial properties in fish culture (Gupta and Sharma, 2006; Dauda *et al.*, 2014) [7]. Phytoestrogenic plants with anti-fertility properties have also been thought of as a solution since they can be easily obtained and incorporated in tilapia feed (Gupta and Sharma, 2006; Dauda *et al.*, 2014) [7].

A study by Haylor and Pascual in 1991 reported successful tilapia sex reversal using ram testes as a source of dietary testosterone. They examined 27 fish and found 23 males and 4 intersex fish. Phelps *et al.*, (1996) [15] obtained a 65% male population using a diet, half of which was freeze-dried bull testes. Another study is the study of Abou Zied *et al.* (2011) [1]. They produced 81% male out of 18,000 Nile tilapia (*Oreochromis niloticus*) using 12.5% of palm pollen grain diet. In recent years, Ghosal *et al.*, (2015) [6] utilized *Basella alba* leaves and *Tribulus terrestris* seeds (0.0, 5.0, 10.0, 15.0 g/kg feed) and immersion treatment with aqueous extracts of both plant materials (0.05, 0.1, 0.15 g/l) to mixed-sex juveniles of Nile tilapia. The treatment with *T. terrestris* aqueous extract at a concentration of 0.15 g/l showed the highest percentage of males (81.4±0.5) during the immersion experiment. Nieves (2017) [14] also utilized Benguet pine pollens (*Pinus kesiya*) and was able to produce 88% of male redirected.

Another indigenous plant that has a potential source of testosterone is black pepper *(Piper nigrum).* Piperine the active constituent of *Piper nigrum* is known to affect testosterone 5α-reductase. This enzyme causes testosterone levels to remain high (Hirata *et al.*, 2007) [9]. The leaves are used photometrically as a contraceptive, antipyretic, antiemetic, carminative, antibiotic, febrifuge, aphrodisiac amongst others (Iwu, 2014) [10]. The leaves of the plant have been demonstrated to exhibit cholinergic activity (Udoh *et al.*, 1996) [19] and a depolarizing neuromuscular blocking action (Udoh, 1999) [18] which may be the basis for its repro-active effects. Pepper also contains phytochemicals, including aides, piperidines, pyrrolidines, and trace amounts of safrole (Citarasu, 2010) [2]. Phytochemicals are also reported to block biosynthesis as well as the action of estrogen by acting as aromatase inhibitors and antagonists to nuclear estrogen receptor and hence may be considered as the potential mean for inducing sex reversal in fish (Gupta and Sharma, 2006; Dauda *et al.*, 2014) [7]. In recent years, Sutyarso and Kanedi (2016) [16] studied the effects of black pepper on the fertility potential of male albino mice. Although such positive claims had not been widely supported by adequate data, these facts should be considered as a scientific challenge.

Considering these aspects, the present study aimed to investigate the potential effect of pepper leaf on the sex redirection of *O. niloticus*, to find the possible concentration that would yield the highest androgenic action and to determine an ideal treatment regime of pepper leaves that will produce a higher percentage of males in tilapia, a growth rate of the stocks and some associated cost and return analysis of using the indigenous hormone

Research Methods or Methodology

Research Design

The study was experimentally done into two (2) phases. The first phase focused on the Range Finding Test designed to determine the range of PLP concentration that would produce a specified number of males. The second phase was the Definitive Test which was designed to determine the concentration of the PLP that would produce the highest percentage of males. In each of these phases, a Completely Randomized Design (CRD) was employed.

Experimental Treatment

Range Finding Test (RFT)

To determine the range of PLP concentration that would produce male fingerlings, different PLP inclusion levels were tested at 1.5% interval per 142 treatment as shown in Table 1**.**

Table 1: Experimental treatments for Range Finding Test (RFT)

Treatment	PLP Test Concentration (% inclusion)	Hormone treatment protocol
1	1.5% PLP inclusion in the feed	PLP dissolved in 200 ml 95% ethanol then mixed with 1,000 g feed; fish fed for 28-days *ad libitum*; 5 weeks nursery rearing for final sex determination
2	3.0% PLP inclusion in the feed	
3	4.5% PLP inclusion in the feed	
4	6.0% PLP inclusion in the feed	

At the end of the nursing period, the stocks were harvested and sex determined manually. The PLP inclusion level with the highest number of fingerlings redirected to male was used as the benchmark for the definitive test run and it was 6.0% or 60g inclusion of PLP per 1 kg of feeds.

Daily ration

For the first 28 days (sex reversal period), the fry were fed *ad libitum* so that they would consume more hormones and the sex was re-directed to male. For the first 28 days, the type of feed used was the fry mash so that it can easily be eaten by the small fry. Then it was changed to a particular size that was appropriate to the growing fish.

Nursery Rearing

After 28 days the fry were transferred to the tanks (size of tank?) of BUTC Wet Laboratory. Stocking density was still the same at 350 fry/tank. They were nursed for five weeks using commercial feed. Feeding frequency was twice a day. After five weeks, ten percent (10%) of the test organisms were sampled to estimate the mean weight and length.

Sampling was also conducted to know its survival rate.

Sex Determination

After nursing, the stocks were harvested and examined individually. The samples that sex was not easily identified were subjected to gonadal examination. Their gonads were examined through a microscope. Tilapia could be sexed with reasonable certainty based on the appearance of the genital papilla if they have not been hormone-treated. But for hormone treated fish, the nature of the gonad does not always correspond with the shape of the papilla. Fish were dissected by making a cut near the anus to below the base of the pectoral fin.

Statistical Analysis

The data obtained from the test were statistically analyzed using the Analysis of Variance (ANOVA) at 0.05 level of significance in determining significant differences among samples exposed to different treatments. Descriptive analysis was also used.

Simple Cost and Return Analysis

The cost and return analysis was used to determine the economic viability of the product under the study. It was conducted to determine the profitability of the product. The total cost and benefits were computed to estimate the potential income and return on investment.

Results and Discussion

Male Redirected

Prior to the conduct of the definitive test, a range-finding test (RFT) was done to ascertain the Pepper Leaf Powder (PLP) inclusion for the definitive test. Results obtained showed that the treatment with 6.0% or 60g inclusion of PLP per kg feeds had the highest percentage of male redirected and thus served as the benchmark for the Definitive Test Run (DTR). As such, 60 g inclusion was used as benchmark level with 0.5% level below and 0.5% level above. The treatments were as follows: T1 with 5.5% or 55g PLP inclusion, T2 with 6.0% or 60g, and T3 with 6.5% or 65g inclusion. Results obtained from the definitive test run are 220 shown in Figure 1.

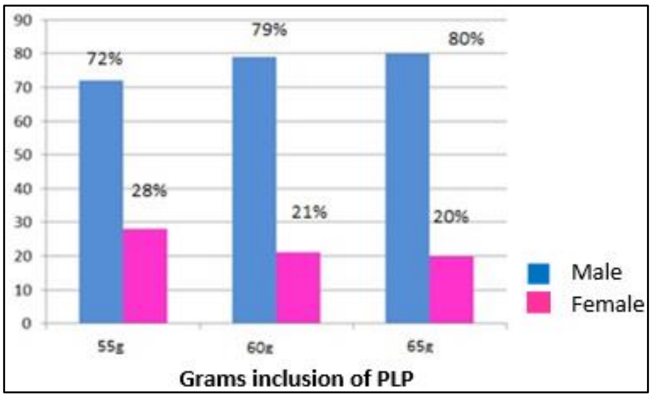

Fig 1: Mean percentage male and female after the Definitive Test Run

Those groups treated with 6.5% PLP inclusion (T3) had the% highest percentage of male redirected (80% male conversion), followed by those with 60 (79 % male conversion) and 55% (72% male conversion) PLP inclusions, respectively. This only seems to suggest a pattern that increasing the PLP inclusion in the feed will increase the number of male tilapia fingerlings redirected or converted. Although this result is lower than what was obtained by Abuo Zied, *et. al.*, (2011) [1] and Ghosal, *et al.*, (2015) [6], it appears that the utilization of PLP is showing some promise. The use of pepper as a phytoandrogen is showing promising effects same with the study of Nieves (2017) [14] when he utilized Benguet Pine Pollens (*Pinus kesiya)* and was able to produce 88% of male redirected for 100% use of the pine pollens. Given, this context, PLP may have a similar effect as17*a*-methyltestosterone which resulted to the 72% to 80 % male conversion.

In this study, a total of 350 fish were stocked and harvested after two months of nursery rearing. Sex determination was done manually. Those not to correspond underwent examination of the gonad. This was accomplished by dissecting the fish and examining the gonad under the microscope. Fish was dissected by making a cut near the anus to below the base of the pectoral fin. The entire gonad, located on the dorsal portion of the peritoneal lining, was removed carefully beginning ventrally and going forward. The gonads were placed on a microscope slide and each given a drop of dye. Another slide is placed on top and gonads were gently rolled or squashed.

Table 2 shows the summary of male redirected by treatment. It clearly showed that treatment 3 with 65% inclusion of PLP got the highest percentage with 80%±13.65 followed by Treatment 2 with 60% inclusion of PLP with 79%±19.04, followed by the first treatment with 72%±19.52 male redirected tilapia.

Table 2: Summary of Male Redirected per Treatment

Treatment	No. of male	Percentage	Std. Dev.	Min	Max	Std. Error
3	235	80%	13.65	219	244	7.88
2	213	79%	19.04	195	233	10.99
1	178	72%	19.52	159	198	11.27

The high percentage of male redirected when fed with PLP revealed a consistent result with the findings of Vijayakumar and Nalini (2006) [20], that piperine supplementation might increase the plasma testosterone level. Piperine the active constituent of *Piper nigrum* is known to affect testosterone 5α-reductase. This enzyme causes testosterone levels to remain high (Hirata *et al.,* 2007) [9]. This property caused anti-fertility to the sexually undifferentiated eyed-eggs tilapia. It is consistent with the result of Dalal and Gupta (2013) [3] when male mice treated orally with black pepper powder significantly showed antispermatogenic and antifertility effects. Such findings supported by many other reports, as reviewed by Ogbuewu *et al* (2011) that the herb plants belong to the pepper family, such as *Piper longum* and *Piper nigrum*, has the potential to be used as a contraceptive material.

The next possibility that makes black pepper affect androgens secretion and the high percentage of male redirected in Tilapia is minerals such as magnesium (Mg) and zinc (Zn). As reported by Bouba (2012), pepper contains magnesium (Mg) in a significantly high concentration. Zinc supplementation can improve the anti-oxidative status and hormone levels in goats by Kumar *et al.* (2013) [12] and proved to increase serum levels of sex hormones including testosterone in rats (Egwurugwu, 2013) [4]. These properties were the reason behind the high positive response of male redirected was shown in the study thereof.

Statistical analysis for the male redirected tilapia for the Definitive Test Run clearly showed that there was no significant difference among the treatment mean at 0.05 level of significance. This simply implies that any of the three treatments can be used since there is no significant difference in the number of male redirected

Growth Rate

Growth response of the fish to different treatments was examined to determine the effects of PLP using the following treatment: T1 with 55g inclusion of PLP, T2 with 60g inclusion of PLP, and T3 with 65g inclusion of PLP. Observation revealed that the presence of PLP in the diet improved fry growth performance (final weight and total gain). Moreover, observation also showed that as the PLP level is increased in the diet, growth also increased with treatment 3 having the highest growth increment among the treatments. This indicates that the level of PLP inclusion in the diet may influence the growth of tilapia fingerlings. The results on weight gain showed in Figure 2 that the highest increment in terms of weight gain was in Treatment 3 (15.64g ± 0.46) with a weight gain ranging from 15.18 g to 15.69 g. This was followed by those fish fed with a hormone-treated feed containing 60 g of PLP (14.16 ± 0.25) followed by treatment 1 with 55g of PLP with 12.26±0.44 which means that its weight ranges from 11.82 g to 12.7 g.

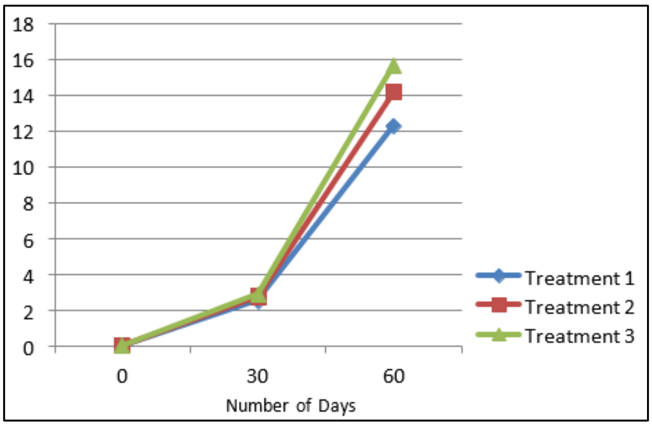

Fig 2: Weight gain of Tilapia Fingerlings using PLP

The average growth increment per day in terms of weight is high at Treatment 3 with 0.25g followed by Treatment 2 with 0.24g then by Treatment 1 with 0.2g. Their average growth upon the yield of Tilapia culture in tanks for 2 months is 0.23g per day. The analysis of variance (ANOVA) of the comparative growth of tilapia fingerlings in terms of weight gain showed that there is a significant difference among the treatment mean.

Figure 3 shows that the highest growth increment in terms of length gain was in Treatment 3 with an average gain of 91.55 mm ± 1.82, which means that the test organisms in Treatment 3 had a length ranging from 89.73 mm to 93.37 mm. The higher mean length gain of the fish could be attributed to a higher amount of PLP of the feed given (65g). The next highest mean length gain was Treatment 2 at 87.74 ± 0.89 and the lowest mean length was 83.24 ± 1.68 in the first treatment.

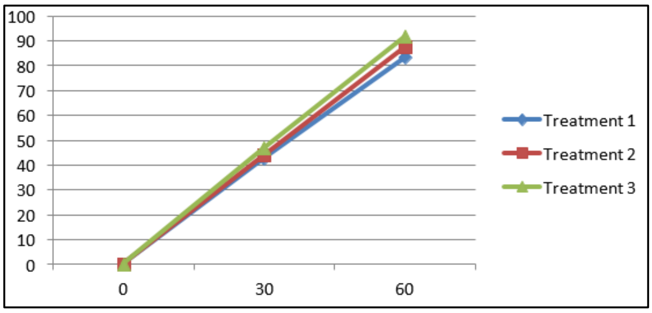

Fig 3: Length gain of Tilapia Fingerlings using PLP

Statistical analysis showed that it is highly significant. This means that the effect of PLP upon the yield of Tilapia cultured in tanks for 2 months differ with each other because 65% inclusion of PLP in the feed is the most effective. This suggests that Treatment 3 showed the highest positive response in terms of length among the other treatments and increasing PLP also increases growth increment in terms of length.

Cost and Return Analysis

The total production cost of the sex redirected tilapia fry was Php 1,497.00. This involved price of the hormone-treated feeds and eyed eggs or newly hatched tilapia. With this amount, the yield was 1,888 pieces that could be sold at Php 1.1 with a weight of 12.55g ± 0.24 and a length of 83.66 ± 1.49. It only costs less than 2 centavos to produce a fingerling treated with artificial sex reversal. Sex-redirected or sex-reversed fingerlings treated with methyltestosterone are sold 5-10 centavos more than non-treated ones. It also showed a net profit of Php 579.8 and gave an ROI of 38.92%.

Conclusion

In the Range Finding Test phase, 60% inclusion of PLP got the highest male percentage with 77.3%. %. It also showed a high growth increment with 12.5g±0.13 and a length of 88.06mm±1.77. Therefore, this treatment served as the benchmark for the next phase of the study. For the definitive test run, three treatments were used: 55%,60%, and 65%. This was based on the result of the range-finding test. Treatment 3 with 65% inclusion showed the highest positive response of male redirected with 80%, followed by 79% and 72% respectively. This shows that increasing the PLP inclusion in the feed also increases the number of male redirected. However, Statistical ANOVA for the male redirected Tilapia for the Definitive Test Run showed that there was no significant difference among the treatment mean. Treatment 3 also showed the highest positive increment in terms of weight gain of (15.64g ± 0.46) which means that it ranges from 15.18 g to 15.69 g. and length gain of 91.55 mm ± 1.82, which means that the test organisms in Treatment 3 had a length ranging from 89.73 mm to 93.37 mm. The cost and return analysis, showed the net profit of Php 1,110.144 and gave an ROI of 38.92%. This means that the use of PLP in the sex redirection of tilapia is profitable

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Statement of Conflict of Interest

The researcher discloses that there's no conflict of interest.

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