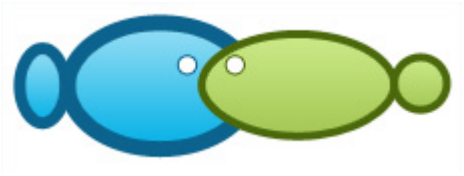

The influence of *Trichanthera* leaf meal on the early development of Nile tilapia (*Oreochromis niloticus*) gonad

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Abstract. The study assessed the condition of the ovary and the ovarian follicles of juvenile Nile tilapia *Oreochromis niloticus* fed with *Trichanthera gigantea* leaf meal (TLM) for a duration of one month reared in an experimental scale aquaponics system. Specifically, the growth was determined by the total body length (TL, cm), body weight (BW, g) ovarian status by the gonad weight (GW, g), gonado-somatic index (GSI, %), oocyte diameter (OD, um), and fecundity (by volumetric method, and approximate number of growing oocytes per ovary). These parameters were supported by histological data on representative ovaries using the standard H&E technique, as indicators of growth and relative gonad maturation in a three-treatment feeding experiment consisting of: pure commerical feed, T0; 50% TLM in commercial feed, T1; and 100% TLM, T2. The feeds were given twice daily with approximately 0.5 g fish^{-1} day^{-1} or 9.0 g/feeding schedule in stocking density of 36 juvenile tilapia per tank (~4 L fish^{-1}). Results after one month showed that relatively smaller juveniles with no significant difference ($p > 0.05$) were in 100% TLM (T2) in terms of TL compared to T1 and to T0 (13.29±1.65 cm, 11.44±0.99 cm, 11.28±0.98 cm, respectively) as well as the BW, which corresponded to TL. The mean female GSI of T2 was significantly higher than T0 and T1 ($p < 0.05$). The estimated fecundity of T2 exhibited direct correlation (R^2) with its TL (0.881), the BW (0.819), and the GW (0.819), which can be attributed to TLM for gonad development as supported by the observed highest fecundity of 2184-7344 oocytes (TL = 11.5-16.3 cm) and mean oocyte diameter (108-504 um). Furthermore, the light microscopic examinations of T2 ovary revealed oocytes at an advanced secondary growth phase (vitellogenic oocyte) with abundant primary growth stage (early and late perinucleolus and yolk nucleus stages). The presence of spent ovaries from relatively smaller sizes of juvenile tilapia fed with pure TLM but not in pure commercial feed or in 50% TLM indicates that the overall ovarian function can be influenced by the feed composition when grown under the controlled aquaponics system. All these observations are promising, which remain inconclusive as they need further physiologic analysis in terms of degree of hepatic vitellogenin expression (mRNA to protein levels) by the liver and of blood estrogen levels under the same condition.
Key Words: aquaponics, ovary, plant-based feed, reproductive biology, tilapia.

Introduction. One of the biggest considerations in rearing fish for efficient production under a highly controlled condition such as aquaculture and aquaponics system is the reproductive capacity of the fish after a given period of maturation. On the contrary, fish gonads and their capacity of producing viable eggs have not been put into priority in most fish farms. However, for the fish culture to be real productive, farmers must provide the best quality feeds that the fish need. As Loum et al (2013) said, the reality of a successful fish culture in tanks requires feeds with the highest budgetary allocation which accounts to 40 to 60% of the total production cost in aquaculture, which makes growing fish in tanks sometimes even more expensive than in the conventional ponds. The compatibility of the close system approach in growing tilapia may or may not have direct or indirect implications to the gonad, specifically the ovaries of the juvenile tilapias. Tilapia remains as the preferred species for tropical and sub-tropical farm conditions (Food and Agriculture Organization of the United Nations 2005; Offem et al 2007) and has been widely grown in inland aquacultural ponds and tested successfully in brackish pond water in mangrove forests, in other words, in a totally different condition of water.

Tilapia (*Oreochromis niloticus*) is the most accessible alternative in every form of study in relation to biology (growth and development) and aquaculture (production) (Abdel-Tawwab 2004) in the fish response to modified environment (Loum et al 2013). The *Trichanthera gigantea*, on the other hand, was introduced in Southeast Asia including the Philippines from Colombia (Vasquez 1987; Perez-Arbelaez 1990) and other Latin American countries (Record & Hess 1972; McDade 1983). It is considered golden for livestock growing (Rosales 1996) by the hog growers and the poultry raisers with nutrient levels already known to many feed manufacturers even before it reached the Philippines, being a potential source of protein, varying from 18-22% in dry matter form and apparently this protein is mostly true protein with good amino acid balance (Rosales et al 1989). This amount of nutrient has great advantage for the gonads of fish (Gaber et al 2012). Hence, this study looked into the impact of nutrient-rich *T. gigantea* leaf meal (TLM) on juvenile tilapia and specifically assessed the growth and the gonadal development after one month of feeding experiment under a highly controlled experimental set-up in aquaponics.

This present work is on juvenile tilapia grown in a small-backyard-scale aquaponics system and experimentally fed with two-level TLM within a nutrient-rich recirculating effluents in a close system to assess the ovarian condition of the juvenile tilapia specifically on growth and development based on GW (g) and TL (cm) correlations, gonadosomatic index (GSI, %), fecundity, and histologic features of the ovary to show advance stages of oocyte development.

Material and Method. A total of 216 randomly selected juvenile Nile tilapia purchased from local hatchery in Initao, Misamis Oriental (Mindanao, Philippines) in May 2014, were randomly distributed into 150L rearing tanks (n = 6) with dechlorinated tap water. Thirty-six fishes were placed in each tank of two replicates of each treatment group as follows: T0, for pure commercial feed (CF); T1, with 50:50 TLM:CF; and T2, with 100% TLM. Feeding rate was set to approximately 1.0 g feed composition $fish^{-1}$ day^{-1} on two-feeding schedules per day for a total of 30 days, from 25 May to 24 June 2014. The pH and TDS (total dissolved solids) were monitored in each tank on a weekly schedule throughout the experimental period.

Total length, body weight, and gonad weight. After 30 days, the total length (TL) and live body weight (BW) were recorded accordingly prior to dissection and sexing. Sexual maturity was determined eventually. The ovaries were carefully detached from the body wall to obtain the fresh weights (g) of the left and right gonads. One of each pair of ovaries from all samples was placed in 10% formalin for histological and light microscopy analysis while the other was kept fresh in ice for teasing and direct microscopic measurement of the intact oocytes.

Gonadosomatic index. All measurements on fish (BW and gonad weight - GW) were used in the determination of the Gonadosomatic Index (GSI, % - Formula 1) based on Gundersen et al (2000), expressed as the percent GW of the fish BW, which can also be used in estimating the age of fish.

GSI % = gonad weight (g) /Body weight (g) X 100 (Formula 1).

Fecundity estimation. Fecundity was estimated using volumetric technique (VT) with use of light microscopy. Using one of the paired gonads from each treatment group to represent the population, the final maturation oocyte stage or oocytes having the largest diameter from each batch were considered, and setting aside third portion (e.g., the posterior, middle, and anterior) of the fresh ovary and carefully released in 70% ethanol, viewed under the light microscope, and the number of ripe eggs was finally counted. The egg size was determined by measuring the diameter of randomly selected eggs per female along two axes using a calibrated eyepiece micrometer and subjecting the images to Image-J software (National Institute of Health 2002). The total number of ripe eggs in the ovary was estimated by multiplying the number of ripe eggs counted from each

ovarian region by the ratio of the ovary weight and finally to the whole ovarian lobe weight. All the data were used to determine regressions for fecundity/length, fecundity/weight, fecundity/ovary weight, and fecundity/egg diameter. The existing relationships between the differences on studied parameters were statistically analyzed by computing the correlation coefficients (r) using Microsoft Excel 2003.

Histological examination. Formalin-fixed (10%) ovary samples from the posterior, middle, and anterior part of gonads were cut separately and dehydrated through ascending grades of analytical reagent (AR) ethanol and cleared for at least 24 hours with xylene, infiltrated, and finally embedded in pure paraffin wax and sectioned using rotary microtome set to 7µm thickness and stained with Haematoxylin and Eosin. The permanently mounted stained sections were digitally photomicrographed using a camera mounted on compound microscope (Motic B5 Professional 3.0, China). Image analysis was performed using ImageJ software (National Institute of Health 2002) calibrated for scale bar and for oocyte measurements.

Statistical analysis. Total length (TL, cm), live body weight of fish (BW, g) (Schneider et al 2000), gonad weight (GW, g), oocyte diameter (OD, µm) (Vazzoler 1996), fecundity (F), and other pertinent information on the biology of tilapia in aquaponics system and fed with different levels of TLM were obtained. Regression analysis was used to determine the following relationships between:

1) Fecundity (F)• with total length (TL); F with live body weight (BW); F with gonad weight (GW); and F with oocyte diameter (OD);

•Fecundity = no. of eggs counted/sampling x 10 x fresh ovary weight (g) x 3 where, 10 was the volume of the solution used;

2) GSI•• with TL; and GSI with BW, where:

••GSI = (Gonad weight, g/fresh BW, g) × 100.

The results were analyzed using regression based on generalized linear models, using a significance level of 5%. These procedures were performed using the software Statistica 7.0®.

Results and Discussion. The length at maturity and the body weight are useful indicators of growth and of the life history of fish in a given environment (Schneider et al 2000; Chu et al 2012; Shafi & Yousuf 2012) and can be greatly influenced directly by the availability of food and other environmental parameters (Shafi & Yousuf 2012) wherein nutrient availability has been linked to pH of water (United States Geological Survey 2014). The prevailing nutrient availability in water with the daily feeding could be realized in the growth response of the fishes within the given period of one month. This means that female tilapia in this tank growth was density-dependent (Tsadik & Bart 2007) so the increase in population size may have led to a decrease in per-capita food availability and thus a decrease in the size at maturity (Bigler et al 1996). However, the environmental conditions can also induce phenotypic flexibility in fishes which leads to changing age at maturity (Wertheimer et al 2004). In this respect, female tilapia fish size at maturity was influenced by the feeding level, which affects the growth of the fishes, which was consistent with the observed effects of dietary energy in the early development of the gonad of sharptooth catfish, *Clarias gariepinus* (Çek & Yilmaz 2009).

Table 1 presents the pertinent growth and development indicators in tilapia over a period of one month in aquaponics set-up. The smallest length recorded during the random sampling was 10.17 from T2 and 15-gram from T1 and T2. For all the fish sampled, the attained mean TLs (cm) of all the representative samples was recorded at 13.29±1.65, 11.44±0.99 and 11.28±0.92, and the mean BWs (g) were at 50.319±11.19, 24.00±4.51, and 26.14±6.38, respectively.

Table 1

Growth and development parameters in nile tilapia (*O. niloticus*) fed diets containing different levels of TLM composition during the rearing days

Parameter	*T0 (Mean±SD)*	*T1 (Mean±SD)*	*T2 (Mean±SD)*
TL (cm)^{ns}	13.29±1.65	11.44±0.99	11.28±0.92
BW (g)^{ns}	50.319±11.19	24.00±4.51	26.14±6.38
GW (g)^{ns}	1.24±0.34	1.35±0.39	1.43±0.34
OD (um)	204.01±90.99	178.18±78.25	359.79±69.11
Fecundity*	3104±1484.55^{a}	4005±1392.11^{a}	4952.14±1510.11^{b}
GSI (%)^{ns}	3.74±1.72	5.63±1.23	5.84±2.42

* significantly different values across treatment groups ($p < = 0.05$); ns – no significantly different values across treatment groups; different letters denotes significantly different values.

The BW in the growing female juvenile tilapia was directly proportional with the TL but was observed to be within the group 20.00 to 40.00g and 10.00 to 14.00cm sizes (Figure 1).

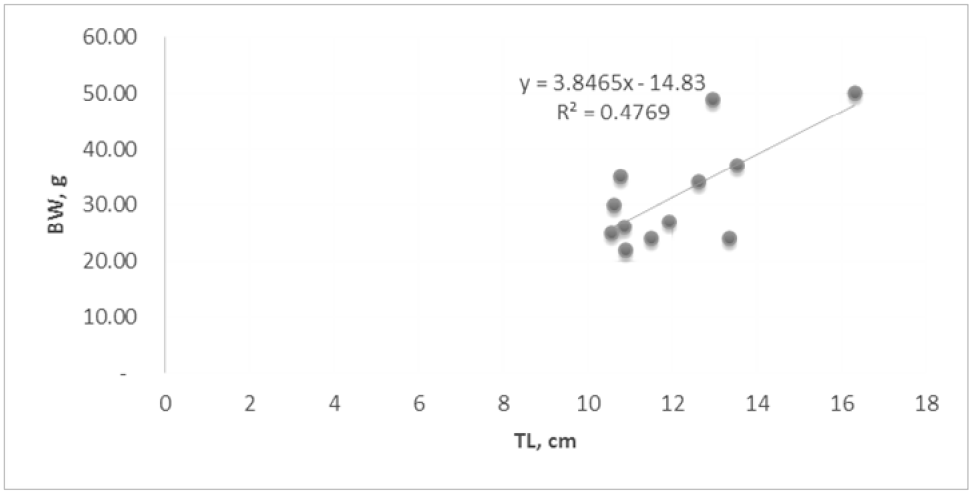

Figure 1. Mean total length (TL, cm) and mean live weight (BW, g) interactions in juvenile *O. niloticus* at the beginning of experimental feeding in aquaponics system. R^2 = 0.4769 indicating direct proportionality between BW and TL in juveniles.

Over a short period of 30 days, juvenile tilapia grown in aquaponics attained a growth rate by weight at 3.00 mg day^{-1} and 0.32 mg day^{-1} for T0 and T2, respectively, while T1 lost weight of about 1.25 mg day^{-1}. The biggest change in BW was observed in T0 (8.18%) and the least was in in T1 which was minus 4.29% of the BW through the 30-day period. This size differences in fish samples used could be explained by the discriminate destructive sampling, wherein, fish caught during sampling could not be intentionally selected – whatever size of fish caught was subject to measurements and dissection. Nevertheless, the tilapia samples used belong to the same batch of fingerlings. These observed growth rates were equivalent to either increment of decrement in the BW of the tilapia in each treatment group. Moreover, a better relationship between the BW and the TL of fish can be observed from bigger population (sample size) and longer observation period (Chu et al 2012). The previous statement can be supported by Bahnasawy (2009) who reported that efficient stocking of tilapia in fertilized tanks can remarkably increase the growth performance and development of the fish.

The starting GSIs of the juvenile tilapias measured one week after the feeding experiment showed low averages between 1.60% to 4.90% with T0 having the least. Thirty days of feeding TLM to T1 and T2 at 50% and 100%, respectively, indicated very minimal differences between the two levels of TLM, which could still be affected by the size of the sample. The T0 group with pure commercial feed appeared to have slightly

exceeded T2 GSI with very small difference of 0.4% and between T2 and T0 with only about 1.0% (Figure 2).

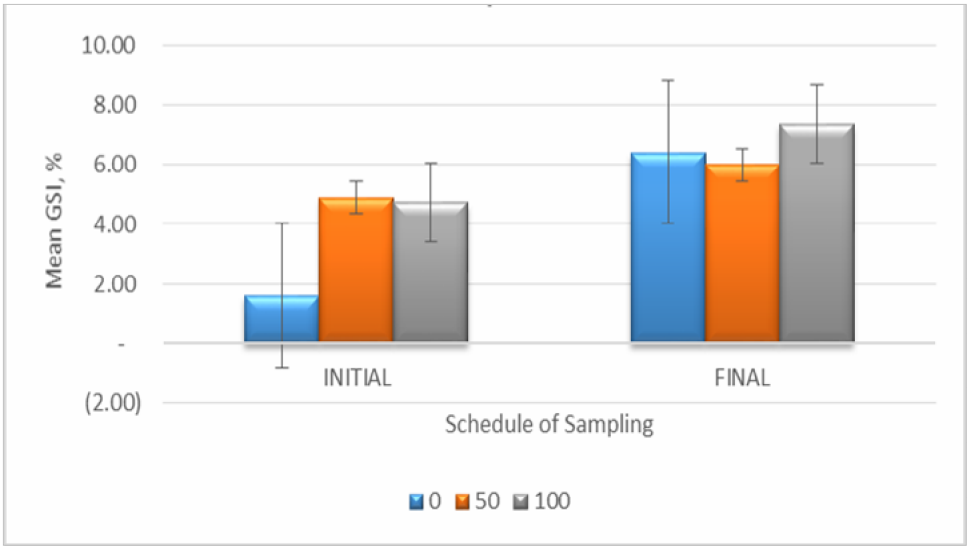

Figure 2. Comparison of the mean gonadosomatic indices (GSIs, %) of female juvenile tilapia before (initial) and after (final) one month of experimental feeding with diffrent levels of TLM (horizontal bars indicate standard errors, se).

The minimal GSI differences between T0 and T2 have implications on the overall condition of the ovaries of the two groups of juvenile tilapias, wherein, it was already not possible to find any ovarian structure which is ribbonlike. The gonads of the tilapia in T2 tanks exhibited fully mature ovaries, which coincided with the observations of Ibim & Sikoki (2014) with the improved protein in diets (40%) of *C. gariepinus*. It could be expected that T2 should have higher GSI values if the tilapias have not spawned yet. However, it was surprising to find spent ovaries in the relatively smaller juveniles (TL, 11.00 cm; BW, 22.00 g) from the tilapia stock, which caused the almost similar GSI values of the two groups of tilapia. This small-sized tilapia with high GSIs can be explained by the still undergoing development in T0 and the spent ovaries in T2 and the presence of residual oocytes (Ndour et al 2013).

The mean relative fecundity of vitellogenic oocytes in the intact ovary of tilapia fed with different feed compositions was recorded at 3.104±1.484.55 (T0), 4.005±1.392.11 (T1), and 4.952.14±1.510.11 for T2 over a period of one month. The obtained values corresponded to fish total length of 11-16 cm and oocyte diameter range of 178-359 um. The fecundity was highly correlated with the total length of fish (r = 0.881), the fresh weight (r = 0.819), the gonadal weight (r = 0.994), as well as with the egg diameter (r = 0.801). The relative fecundity obtained in this present work on tilapia is comparable to the relative fecundity of *Rasbora tawarensis* having spent (Stage V) ovaries (Muchlisin et al 2011).

The low fecundity of the T2 group was not of similar condition to T0. While T0 did not show any complete maturity of the gonad, the T2 group exhibited fully grown ovaries and even spent ovaries (n = 2). The female fecundity of tilapia was better correlated with the TL and showed that as TL increased, the fecundity also increased in all the feeding compositions. Most of the parameters to show the growth and development of the gonad of female juveniles appeared to be inconsistent with the levels of TLM throughout the 30-day period of feeding experiment in aquaponics system. The GW and the GSI are expected to coincide, wherein both parameters were highest in T0 and lowest in T1. The fecundity did not follow the same trends based on the levels of TLM. The most fecund among the groups was the T1 which could be due to the presence of unspawned eggs during the sampling schedule. The mean oocyte diameter of tilapia from 100% TLM exhibited largest oocytes while T1 and T0 both have the smallest oocytes.

Squashed gonad and histological examination of juvenile tilapia. The various stages of growing oocytes in intact ovarian tissues of juvenile tilapia with the corresponding histologic and morphologic states of the oocytes are shown in Figure 3. The wide range of oocyte diameter in the ovaries of tilapia that received pure TLM within a 30-day period indicated a more advanced stage of gonad development. This is supported by the presence of large yolky oocytes with signs of the germinal vesicle breakdown and the more dispersed yolks within the cytoplasm.

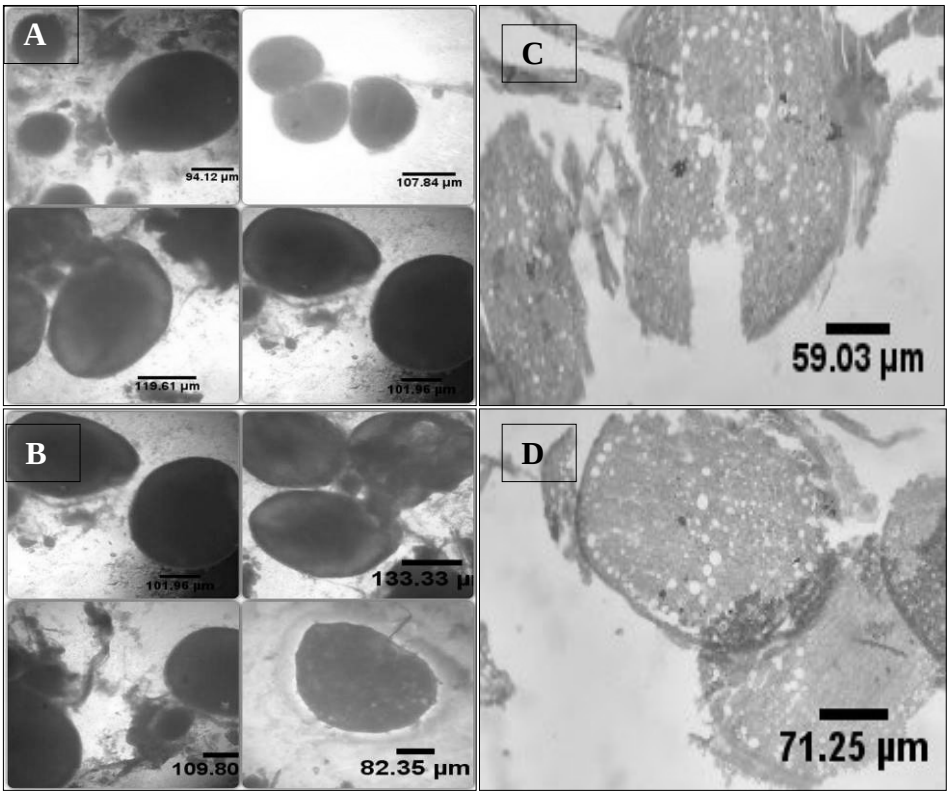

Figure 3. Large yolky oocytes (~diameter, 178-360 um) used in determining the fecundity (A, B) from the freshly teased ovary in 70% ethanol showing the different densities of the cytoplasm (light and dark) and the histologic features of gravid ovary showing advanced stages of oocyte development with eosinophilic cytoplasm and smaller oil globules (empty vesicles) (C, D) in juvenile tilapia grown in aquaponics and received 100% TLM as feed (T2).

The different developmental stages of the oocytes were observed in this study from all the treated groups. Oocytes undergoing sequential changes through oogenesis (Zaki & EL-Gharabawy 1991) were documented in both fresh and fixed samples. Microscopic examination indicated that many stages of oocytes were consistently present from all treatment groups. The ovary samples from T0 group initially showed abundant loose connective tissues with oocyte sizes that are not that obviously visible (not shown). The T1 group, on the other hand, was already showing yolk globules but the germinal vesicles are not yet visible. Tilapia is known to be a multispawning fish and it appeared to become more efficient multi-spawning when fed with *Trichanthera* leaves. Histologic sections from T2 group have shown direct influence on the growth and development of the gonad enabling the juvenile tilapia oocytes to undergo germinal vesicle migration (GVM) and breakdown (GVBD), which are indications of final oocyte maturation (FOM) and with mature or ripe ovaries (Stage V), similar to the effects on the gonads of *Clarias gariepinus* using high crude protein in the diet (Ibim & Sikoki 2014) and in tilapia on different commercial feed additives (Abdelhamid & Mehrim 2014).

The small oocytes of the ovaries from T2 are of more or less spherical-shaped cells rich in cytoplasm. In the more advanced stages, these oocytes become larger and with the number of nucleoli arranged along the inner side of nuclear membrane (Stages III and IV) (Srijunngam & Wattanasirmkit 2001; Wafaa et al 2011; Gaber et al 2012). In

some cases, the nucleus is large and surrounded with increased mass of cytoplasm appearing less basophilic but not showing any form of damages to the ovarian organization, which can be observed in Neem-fed tilapia at higher dietary level of 2.0 g kg^{-1} (Jegede & Fagbenro 2008). The normal follicles are covered by a layer of simple squamous lining surrounding the oocyte. At even more advanced stages, the cortical alveoli formation stage is characterized by the appearance of clear vesicles (cortical alveoli) in the cytoplasm (Garcia et al 2001; Srijunngam & Wattanasirmkit 2001; Wafaa et al 2011) and a thin acidophilic zona radiata or primary envelope becomes visible (Abdelhamid & Mehrim 2014). The follicles consist of simple cuboidal or columnar layer surrounded with stratified squamous thecal layer, where most of this stage were common in T1. Further development of the oocyte results to a vitellogenic (yolk) stage, when the oocyte size greatly increases and small yolk granules become visible as a ring of deep eosinophilic structures (Srijunngam & Wattanasirmkit 2001) in the cytoplasm and later incorporated in the whole cytoplasmic area. This is similar to the observations of Abdelhamid & Mehrim (2014) after feeding tilapia with commercial additive; the nucleus can be observed to be still convoluted and the zona radiata as a clearly visible noncellular deep eosinophilic band. When the oocytes become fully mature, as was observed mostly in T2, the oocytes are characterized by the enlargement of both cortical alveoli and the yolk granules and the oocytes are markedly increased (Garcia et al 2001; Srijunngam & Wattanasirmkit 2001; Wafaa et al 2011). The peripheral migration of the nucleus can be observed and the zona radiata became more clearly visible, which appeared mostly in T2. These observations in the ovarian development of T2 tilapia coincided with the results from the work of Abdelhamid & Mehrim (2014) which received different compositions of commercial feed additives. However, the advantage of using natural plant-based feed that can be expected with the utilization of *Trichanthera* leaves was the surprising early female and male spawners in T2 group (n = 2) and embryo sacs were collected from the mouth of the two females in the group. These are signs of better reproductive performance and higher gonadal maturation similar to the observations of Ibim & Sikoki (2014) in the improved crude protein levels in the diet of African catfish.

The true proteins (from the crude protein content of 15 to 22%) present in the leaves of *Trichanthera* can provide the amino acids needed in the maturation of female gonad, as well as the calcium it contains in the cystoliths, common in acanthacids, found to be particularly high compared to other fodder trees (Rosales & Galindo 1987; Rosales et al 1992). Moreover, the carbohydrate fraction of the TLM is mostly composed of water soluble carbohydrates and the total and reducing sugars were higher when compared with other fodder trees and shrubs, which may explain its compatibility with the monogastrics, with great implications on the ovarian development of juvenile tilapia (El-Sayed & Kawana 2008).

Conclusions. The nutrient-dense *Trichanthera* leaf meal has influenced the ovarian development of the juvenile tilapia grown in aquaponics system based on the relative fecundity and the oocyte maturation. Histological analysis of the mature ovaries of tilapia under the 100% *Trichanthera* leaf meal showed advanced oocyte maturation and possibility of early spawning. Fully substituting the commercial feed with the leaf meal of *Trichanthera* can be beneficial in tilapia farming.

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