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Do ethinylestradiol (EE2) and levonorgestrel (LNG) affect fish energy reserves? Insights from female guppies

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ABSTRACT. Contraceptive pills containing 17α-ethinylestradiol (EE2) and levonorgestrel (LNG) can contaminate aquatic environments and affect fish physiology. However, studies on the effects of EE2 and LNG on fish physiology in Indonesia remain limited. This study aimed to investigate the effects of combined EE2 + LNG and fish feed on the energy reserves, specifically lipid and protein content, in female guppies (*Poecilia reticulata*). Fish were reared for seven days with or without feeding in 4-l aquaria containing 1 l of water (pH = 7.6 ± 0.06, temperature = 24.6 ± 2.08 °C, BOD = 5.8 ± 0.76 mg l^{-1}). Each aquarium contained five female fish (2–2.5 cm in length). Five EE2 + LNG concentrations were tested: 0 + 0, 3 + 15, 30 + 150, 300 + 1500, and 3000 + 15000 ng l^{-1}, with three replicates. Lipid and protein contents were estimated gravimetrically and expressed in Joules (J). Data were analyzed using two-way ANOVA with EE2 + LNG concentration and feeding as factors, followed by Tukey's post hoc test. Results showed no significant interaction or single factor effect of EE2 + LNG concentration and feeding on dry weight, lipid, or protein contents. Dry weight, lipid, and protein ranged from 17.8–118.0 mg, 0.01–0.49 J, and 0.29–1.94 J, respectively. Thus, exposure to EE2 + LNG did not affect the energy reserves of female guppies. This indicates that short-term exposure to EE2 and LNG at concentrations relevant to the environment does not lead to immediate disruption of energy metabolism in fish, and it also offers reference information for environmental surveillance and toxicological risk evaluation of endocrine-disrupting pharmaceuticals in water systems.

Keywords: contraceptive pills; ethinylestradiol; levonorgestrel; lipid content; protein content

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INTRODUCTION

Environmental pollution caused by harmful chemicals has the potential to be toxic to living organisms. Several studies indicate a lack of information in the literature regarding the toxicological effects of drugs on aquatic organisms (Fent *et al*., 2006; Chen *et al*., 2021; Jureczko & Przystaś, 2024; Martin *et al*., 2025). Drug-related pollution can have harmful effects on aquatic life because aquatic organisms are considered highly susceptible to sewage contaminants due to their direct exposure to these pollutants in their environment (Matthee *et al*., 2023; Pinkey *et al*., 2024). One of the contaminants found in water bodies is contraceptive drugs, namely birth control pills (Weerasinghe *et al*., 2023).

Pollution caused by contraceptive drugs may arise from several sources, such as the disposal of medical waste from healthcare facilities, the disposal of unused drugs through the toilet, or the disposal of the human body's excretory system (Freitas & Radis-Baptista, 2021; Karungamye *et al*., 2022). Such discarded or released contraceptive drugs will pass through liquid waste treatment plants, which are not designed to handle these contaminants. The estrogenic hormone or contraceptive material that is released is often found through sewage systems, passing through waterways, and ultimately accumulate in aquatic habitats. Furthermore, in some communities, it is said that access to the oral contraceptive pill was provided to fish for farming. The practice is premised on the belief that hormones found in contraceptive pills lead to weight gain by causing fluid retention and fat deposition (Gallo *et al*., 2014; Lopez *et al*., 2016).

Based on this conviction, certain fish farmers include contraceptive pills in the fish feed to promote fish growth and body weight. These practices might also lead to the discharge of pharmaceutical substances into the aquatic milieu, and thus to an increased exposure of farmed and wild fish to endocrine disrupters. 17α-ethinylestradiol (EE2), a strong synthetic estrogen found in contraceptive pills, is already known to contaminate water bodies and interfere with fish reproduction and endocrine functions (Weerasinghe *et al*., 2023). These substances are commonly found in water environments and are considered as emerging pollutants due to their stability and biological effectiveness (Grzegorzek *et al*., 2024; Wang *et al*., 2025). Synthetic estrogens such as EE2 can cause changes in reproductive organs and tissues, and affect reproductive processes such as development and maintenance of the female reproductive system (Gonsioroski *et al*., 2020; Wojnarowski *et al*., 2021). It has also been stated that lipid metabolism and energy metabolism processes might be affected by estrogen exposure, meaning that there might be an influence on the energy reserves of the organism (Son *et al*., 2023).

Combined oral contraceptive pills are usually composed of an estrogen and a progestin (Gandhi & Supe, 2024). A commonly used estrogen is ethinylestradiol, which is given at doses of less than 50 µg per tablet (Cooper & Patel, 2025). Levonorgestrel (LNG), a synthetic progestin, is also a major ingredient in many contraceptive products, such as oral tablets, intrauterine devices, and subdermal rods (Hofmann *et al*., 2020). Environmental monitoring studies have reported levonorgestrel (LNG) in surface waters at 38 ng l^{-1} in Malaysia, 33 ng l^{-1} in France and 22.2 ng l^{-1} in China (Liu *et al*., 2011; Al-Odaini *et al*., 2013; Creusot *et al*., 2014). In experimental studies, it has been shown that low concentrations of LNG may influence aquatic biota (Shi *et al*., 2021), reporting the lowest observed effect concentration (LOEC) for zebrafish (*Danio rerio*) sex differentiation at 5.5 ng l^{-1}.

LNG treatment was demonstrated to cause strong androgenic and anti-estrogenic effects in fish such as altered reproductive cycles and secondary sexual characters (Svensson *et al*., 2013). Exposure to synthetic estrogens and progestogens also led to changes in gonadosomatic index, lipid metabolism, and protein production in climbing perch (*Anabas testudineus*) (Weerasinghe *et al*., 2023). In addition, protein metabolism and physiological status in vertebrates are known to be affected by hormonal contraceptives (Dordevic *et al*., 2025; Khan *et al*., 2025). However, the data on the joint impact of EE2 and LNG on energy reserves, especially in tropical fish like guppies (*P. reticulata*), is still scarce. Most of the studies conducted have been on reproductive and endocrine end points; potential effects on energy allocation, including lipid and protein reserves, are not well understood.

Birth control pills that enter the environment and contaminate the habitat of aquatic organisms affects the survival of fish and physiological condition. Previous studies have shown links between energy reserves and gonadal growth in rainbow trout (*Oncorhynchus mykiss*) and climbing perch (Bhat *et al*., 2022; Weerasinghe *et al*., 2023). Fish need lipids as the main source of energy for reproduction and also the maturation and development of eggs during the reproductive process of female fish (Ladisa *et al*., 2022). Lipid content fluctuations that occur in the ovaries, testicles, and liver lead to variability in the gonadomatic index (Bhat *et al*., 2022; Zhang *et al*., 2026). Ethinylestradiol combined with levonorgestrel has an effect on the release rate of the hormone levonorgestrel with lower concentrations of sex hormone-binding globulin in zebrafish gills (Miguel-Queralt & Hammond, 2008). Low concentrations of sex hormone-binding globulins can lead to an increase in free androgen serum that leads to ovarian dysfunction in zebrafish (Wang *et al*., 2020). Nonetheless, studies on the effects of ethinylestradiol and levonorgestrel on guppy fish (*Poecilia reticulata*) in Indonesia is still very limited.

Guppy fish is a freshwater fish that lives in waters in the tropics and is often cultivated (Beanal & Nugroho, 2025). The ability of guppy fish to adapt and reproduce facilitates its distribution in large freshwater waters (Sasanami *et al*., 2021). The male guppy fish has an attractive color and tail pattern with its short body, whereas the color and pattern in female guppy fish does not look attractive and has a rounded body that is longer than the male's body (Sato & Kawata, 2020; Herdegen-Radwan *et al*., 2021). This fish is one of the types of fish that has high tolerance and adaptability so that it can be used as a model organism in toxicology studies (Gomes-Silva *et al*., 2020). Until now, there have been no reports on the effect of the combination of ethinylestradiol and levenorgestrel on the energy reserves of female guppies in Indonesia.

Although there is increasing evidence of the endocrine-disrupting properties of synthetic hormones, data on their effects on energy stores in tropical fishes are scarce, especially at the short-term exposure. Energy stores like lipids and proteins play an important role in fish physiology and are linked to growth, reproduction, and survival. Nonetheless, it remains to be established whether exposure to a mixture of EE2 and LNG, during feeding and fasting, influences energy budget in fish.

This investigation is designed to assess the influence of EE2 and LNG exposure on energy stores, in terms of lipid and protein content, in female guppies (*P. reticulata*). It is hypothesized that EE2 and LNG exposure may influence the energy reserves by causing changes in the lipid and protein content especially in different feeding regime, that might result in altered energy allocation. The present investigation results are anticipated to enhance our understanding of the physiological responses of tropical fish to pharmaceutical contaminants and to provide baseline data for ecological risk assessment.

MATERIALS AND METHODS

Each aquarium with a volume of 4 l was filled with 1 l of well water that has been allowed to settle for 48 hours. Before the experiment, the parameters of pH, temperature, dissolved oxygen (DO), and biological oxygen demand (BOD) of water were observed. Water temperature (°C) and water pH were measured using a pH measuring pHep instrument (HANNA). DO and BOD were measured using the titration method.

To measure DO, well water samples were collected and put into dark winkler bottles slowly to avoid the formation of air bubbles following standard procedures described by APHA (2017). Subsequently, 1 ml of $MnSO_4$ was added, followed by 1 ml of alkaline iodide was added until a precipitate formed (Creusot *et al*., 2014). After the precipitate developed, 1 ml of H_2SO_4 was added and the bottle was shaken gently until the precipitate completely dissolved. The treated water sample was then transferred into an erlenmeyer flask. Titration was carried out using 0.025 N NaS_2O_3 on a 100 ml of aliquot of the samples until a light yellow color was formed. Two drops of starch indicator were then added and titration was continued until the blue color disappeared.

To measure BOD, water samples were collected and put into two 100 ml winkler bottles (DO_0) according to APHA (2017). Another two 100 ml winkler bottles (DO_5) were filled with the same sample and each was inoculated with 4 ml polyseed using a measuring pipette. The DO_5 bottles were incubated for 5 days at 20 $\pm$ 1 °C. After incubation, 0.33 ml of $MnSO_4$ solution and 0.33 ml of alkaline iodide azide solution were added to each bottle using micropipettes. Once the precipitate has settled, 1 ml of concentrated H_2SO_4 was added and the bottles were gently mixed until the precipitate fully dissloved. The sample was transferred into a 50 ml measuring flask and titrated with 0.025 N $Na_2S_2O_3$ until the color changed to light yellow. An amylum indicator was then added, and titration was continued until the blue color disappeared. The titrant volume was recorded and all measurements were performed in duplicate (Jouanneau *et al*., 2014). BOD_5 was calculated as the difference between initial dissolved oxygen (DO_0) and dissolved oxygen after five days of incubation (DO_5).

Healthy and active adult female guppies (2 - 3 cm in length) were acclimatized in the laboratory in an aquarium filled with water for 5 days in 12/12 hours of light/dark cycle. During this period, fish were fed commercial fish feed in the morning and evening at 3-5 % of their body weight (Lucon-Xiccato & Dadda, 2016).

After acclimatization, fish were randomly distributed in 4 l aquaria containing 1 l of water, with five fish per aquarium. The experiment consisted of two feeding conditions: one group was fed and the other group was not fed. Five concentrations of a mixture of ethinylestradiol and levonorgestrel were tested, each with three replicates. Each treatment therefore consisted of 15 fish (five fish per aquarium × three replicates). The birth control pills are dissolved in well water according to the predetermined concentration of EE2 + LNG, namely 0 + 0 ng l^{-1}, 3 + 15 ng l^{-1}, 30 + 150 ng l^{-1}, 300 + 1500 ng l^{-1}, and 3000 + 15000 ng l^{-1}.

The energy reserve (J) in the form of fat and protein content in guppy fish were determined gravimetrically following Reznick (1983). On the seventh day of exposure to the birth control pills, fish were sampled to assess their energy reserves. Each fish was dried at 60 °C in an oven (Memmert) overnight. The fish sample was then placed in a desiccator for 30 minutes. To obtain a mass 1, the fish was weighed to the nearest 0.1 mg.

Fish samples were extracted using n-hexane in a Soxhlet apparatus at 68-70 °C for 30 extraction cycles (approximately six hours) to remove triglycerides until constant mass was achieved. Complete extraction was indicated by a clear and colorless solvent. Extracted samples were again dried at 60 °C overnight, then placed in a dessicator for 30 minutes. To obtain mass 2, each extracted individual was weighed to the nearest 0.1 mg.

Prior to use, porcelain crucibles were dried at 105 °C for 24 hours, then cooled in a dessicator for 30 minutes, after which the dry weight of the empty crucible was measured and recorded. Individual fish that have been extracted with known dry mass were placed into the crucibles. The fish were ashed at 550 °C in a furnace (Thermolyne). After three hours, the furnace was turned off and allowed to cool overnight. The crucibles containing the fish ashes were dried at 105 °C for 24 hours and cooled in a desiccator for 30 minutes. The ash was weighed to obtain mass 3. The difference between mass 1 and mass 2 was the fat content of those tissues and was multiplied by 9.5 to convert to calories. Mass 2 minus mass 3 was the protein content and was multiplied by 5.7 to convert to calories. Units of calories were converted into joules by multiplying by 4.184. Carbohydrates in lean tissue were considered negligible (Reznick, 1983). All animal testing, handling, and experimental procedures followed the principles of the Helsinki Declaration. Ethical permission for this research has been received from the UKSW Research Ethics Commission (ethics reference number 045/KOMISIETIK/EC/9/2023).

RESULTS AND DISCUSSION

The results of the water quality measurement at the beginning of the experiment showed a pH value of 7.7 $\pm$ 0.06 and a water temperature of 24.6 $\pm$ 2.08 °C. Biological oxygen requirement was 5.8 $\pm$ 0.76 mg l^{-1}. The influence of EE2 + LNG exposure on dry weight, lipid content, and protein content in female guppies under fed and unfed conditions is shown in Fig. 1. In general, there was no obvious pattern (i.e., concentration-dependent response) that could be discerned among treatments in both feeding condition. This means that differences were consistent low between each treatment level and not systematically increasing or decreasing with exposure levels.

Dry weight values in both fed and unfed individuals varied little over all treatments and were about 0.04 – 0.06 g. The fact that these numbers are similar for all concentrations indicates that EE2 + LNG exposure did not induce a significant loss or gain of body mass under the experimental conditions. Lipid content also did not consistently vary with EE2 + LNG levels. Lipid content in the fed group fluctuated between about 0.13 and 0.19 J and that in the unfed group between about 0.04 and 0.06 J. Even though clearly different between feeding conditions, the exposure concentrations within each group showed quite some overlap, indicating that the observed difference was not a result of EE2 + LNG exposure.

A similar trend was observed for protein content: approximately 0.69 – 1.05 J (fed group) and 0.72 – 1.00 J (unfed group). The overlapping ranges across treatments also imply that protein-based energy reserves were probably not influenced by the concentrations of EE2 + LNG tested. Taken together, these findings corroborated the statistical analyses and suggest that short-term exposure to EE2 + LNG did not result in detectable alterations of energy reserves in female guppies.

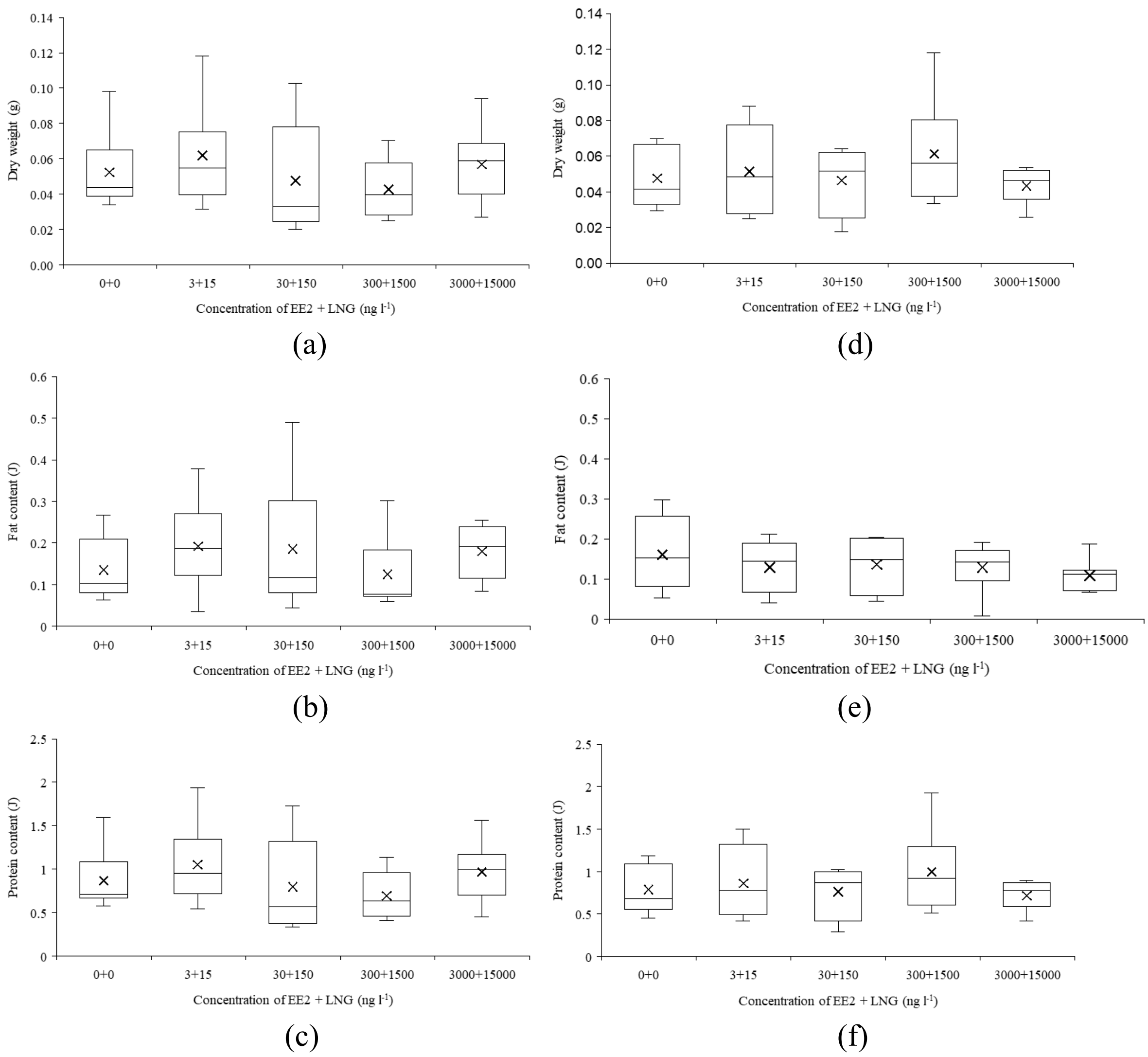

Fig. 1. Box plots of dry weight (g), fat content (J), and protein content (J) in female guppies (*Poecilia reticulata*) exposed to contraceptive pills containing EE2 + LNG under fed and unfed conditions. Panels (a-c) represent the fed group, while panels (d-f) represent the unfed group. Different EE2 + LNG concentrations are shown on the x-axis

The results of this study indicate that EE2 + LNG exposure did not have a significant impact on female guppy dry weight, lipid or protein under the test conditions. This observation corroborates with other studies that describe negligible physiologic effects of short duration exposure to endocrine-disrupting compounds in the environment at concentrations consistent with local environmental levels.

At the molecular level, EE2 and LNG bind with high affinity to estrogen and progesterone receptors, the key transcription factors that control the expression of genes involved in reproduction, development, and metabolism. Treatment with EE2 has been associated with modulation of expression of estrogen receptor genes and their downstream targets that participate in endocrine and metabolism regulation (Hultman *et al*., 2015; Ke *et al*., 2022). However, such responses at the transcriptional level may not immediately result in changes in the monitored parameters of whole-body energy stores in short duration exposure experiments.

Moreover, endocrine-disrupting compounds (EDCs) can affect lipid metabolism by activating or inhibiting specific signaling pathways (e.g., AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR)), which modulate lipogenesis and the systemic energy homeostasis. Environmental estrogens have been shown to modify lipid profiles in fish via these pathways and induce metabolic changes as opposed to more direct physiological responses (Sun *et al*., 2020). Fish may also achieve energy homeostasis by other compensatory mechanisms. Estrogenic agents have been shown to affect associated metabolic pathways including lipogenesis, fatty acid transport, and glucose metabolism, but these changes may be blunted by other regulatory mechanisms that help keep total energy reserves stable (Wojnarowski *et al*., 2022).

Another explanation is that energy stores are a relatively coarse organismal-level output. Although endocrine-disrupting compounds may induce rapid changes in hormone signaling and gene expression, alterations in lipids and proteins composition generally need longer exposure times to accumulate and be measurable. For example, estrogen exposure has been demonstrated to modulate growth-regulating endocrine mechanisms including the GH/IGF axis without inducing immediate changes in organismal growth or biomass (Alcaraz *et al*., 2021; Canosa & Bertucci, 2023).

In addition, the concentrations applied in this study, while environmentally relevant, might have been too low to provoke measurable metabolic disruption. Energy-related whole-body parameters tended to be less sensitive to the two compounds than reproductive and molecular endpoints (Vilaça *et al*., 2024). Energy reserves seemed to be also affected by feeding regimes (fed vs. unfed), as energy reserves varied among groups, pointing to a possible stronger effect of nutritional status on the metabolic outcome than short exposure to EDCs.

Taken together, these results suggest that EE2 and LNG may interfere with endocrine signaling pathways at the molecular level, but their impact on energy metabolism can be masked by compensatory mechanisms and that more/longer exposures or more sensitive endpoints may be needed to detect such effects.

However, some studies have shown no effect of birth control pills on fish physiology. For example, rainbow trout exposed to 10µM EE2 + 10µM LNG had a pronounced estrogenic effect on RTL-W1 cells without significant whole-organism responses (Vilaça et al., 2024). Genes related to lipid and peroxisome metabolism in brown trout (*Trutta psalm*) were not significantly affected by EE2 exposure at environmental concentrations (1-100 ng l^{-1}) (Alves *et al*., 2024).

Although the study showed no significant effect on biomass and energy reserves in female guppies, these results are still important given that the presence of estrogenic compounds such as EE2 has been reported in Indonesian waters, including the Bengawan Solo and Brantas rivers (Ismanto *et al*., 2022). Chronic exposure to endocrine-disrupting chemical compounds in these rivers may have implications for the health of wild fish and potentially reduce the quality of aquatic ecosystems. The results of studies showing that fish communities in Indonesian river waters have recovered after a decrease in contaminants confirm that endocrine-disrupting chemical compounds play a role in suppressing the condition of fish communities (Samosir *et al*., 2024). Thus, although this study did not find significant changes in biomass and energy reserves within 7 days, the findings remain relevant as a basis for understanding the potential risks of EE2 + LNG in fish in Indonesia.

Taken together, this study showed that acute exposure to EE2 + LNG was not enough to alter energy reserves in female guppies, whereas previous studies have shown a noticeable effect on chronic exposure. Follow-up research needs to be conducted over a longer period (≥ 21–28 days) as well as by measuring molecular biomarkers (e.g., vitellogenin, lipid metabolism genes, and liver signal transduction pathways) to detect early effects that may not be reflected in biomass. Additionally, analysis of bioaccumulations in tissues can help understand the relationship between environmental exposure and potential physiological impacts.

CONCLUSION

This study provides evidence for the lack of effect of short-term exposure of female guppies to EE2 and LNG at concentrations that are relevant to the environment on the energy stores. Results imply that energy metabolism-related parameters, i.e. lipids and proteins, could be under the influence of EDCs but this effect might be difficult to detect after short-term exposure. These findings also emphasize the need to examine the influence of exposure time and endpoint sensitivity in pharmaceutical contaminant ecological risk evaluation. Further research should focus on the long-term effects of exposure and on the use of more-sensitive biomarkers (hormonal responses or gene expression) to improve the understanding of the impact of EE2 and LNG on aquatic organisms.

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