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CONTENT

Heat and Water Vapor Production in Farm Animals

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ABSTRACT

Generally, when heat-humidity balance calculations are made in animal production structures, the total heat, sensible heat, latent heat and water vapor amounts emitted by the animals to the shelter environment are taken as basis. Heating is not done unless absolutely, necessary. If there is a heat deficit, the first step is to insulate the building elements. On the other hand, when designing ventilation systems in shelters, the minimum and maximum capacity of the system is tried to be determined based on sensible heat for summer conditions and latent heat or water vapor amounts for winter and transition seasons. In this context, first of all there is a need to know the equations used in calculating the amounts of heat and humidity emitted by farm animals to the shelter environment under project conditions and the data obtained from studies conducted in this field. Within the scope of this study, the equations, principles and data used in calculating the amounts of heat and water vapor emitted by farm animals of various breeds and races were brought together with literature research.

Key words: Farm animals, Total heat, Sensible heat, Latent heat, Heat-humidity balance

1. INTRODUCTION

Increasing the income from animal husbandry within economic limits is possible by improving the genotype and keeping the environment at appropriate levels. Farm animals radiate heat and moisture to the environment they are housed in. The amount of heat and moisture they radiate varies depending on the temperature-humidity values of the environment, and the housing, feeding and watering systems.

In farm animals, body temperature is stable and varies within very narrow limits despite large changes in ambient temperature. Normal body temperatures vary depending on species and rearing direction, and are approximately 38 °C in farm animals such as cattle and 41.7 °C in poultry such as chickens (Bolukbası, 1989; Mutaf, 2012). Keeping body temperature within the limits mentioned is possible by balancing the heat production and heat emission from the body. In cold environmental conditions, it increases metabolic heat production, while keeping heat loss at low levels. In hot environmental conditions, it decreases metabolic heat production, while increasing latent heat emission (Stowell et al., 2001). Body temperature is kept constant by metabolic heat production and heat transfer between the body and the environment, and varies within very narrow limits depending on the time of day, mobility, feeding and thermal environmental conditions. Heat transfer

between the living being and the environment occurs as a result of the temperature and vapor pressure difference between the living being and the environment. The continuity of the stasis in body temperature is possible by ensuring the balance between the heat gain in the body and the heat emission from the body. When the body temperature is higher than the ambient air temperature and the inner surface temperature of the surrounding structural elements, there is a continuous heat loss from the body to the external environment in winter and transition seasons, and in the opposite conditions, in summer, there is a continuous heat load from the external environment to the body (Mutaf, 2012).

Generally, when heat-humidity balance calculations are made in animal production structures, the total heat, sensible heat, latent heat and water vapor amounts that animals emit to the shelter environment are taken as basis. Heating is not done unless absolutely necessary. If there is a heat deficit, the insulation of the structural elements is primarily used. On the other hand, when designing ventilation systems in shelters, the minimum and maximum capacity of the system is tried to be determined based on sensible heat for summer conditions, and latent heat or water vapor amounts for winter and transition seasons (Kocaman et al. 2007). In particular, the selection of project criteria regarding the temperature and relative humidity of the region and the determination of their effects on structural features are of great importance. The aim in determining the project criteria should be to provide an economically appropriate balance between the inside and outside of the shelter that will not create heat and cold stress in the animals housed.

In livestock farms, the ability to control the climatic environment in shelters within economic limits and to keep animal welfare in the comfort zone is only possible by knowing the amount of heat and water vapor that animals emit to the shelter environment. This study was conducted to present the parameters and equations used in calculating the amount of heat and humidity emitted by farm animals kept in shelters in an orderly manner with detailed literature research and to provide an infrastructure for researchers who will work on this subject.

2. KEEPING BODY TEMPERATURE STABLE IN FARM ANIMALS

In farm animals, body temperature is stable and varies within very narrow limits despite large changes in ambient temperature. In keeping body temperature stable, metabolic heat gain in the body is mainly provided by the nutrients consumed, and we can group these into three main groups: proteins, fats and carbohydrates. The chemical means of keeping the body temperature constant with metabolic heat production. The metabolic rate varies depending on the animal's species, sex, body size or body surface area, health status, age and thermal environment. In addition, depending on the thermal environmental conditions, there is also heat gain from the external environment by conduction, convection and radiation. Heat loss is also in two main ways, the first is sensible heat transfer by conduction, convection and radiation; the second is latent heat transfer by evaporation from the body surface as a result of sweating and respiration. Keeping the body temperature constant is achieved physically by transferring sensible heat and latent heat (Mutaf, 2012). The body temperature change limits of some farm animals are given in Table 1 (Bolukbası, 1989; Mutaf, 2012; Kocaman and Kurc, 2021).

Table 1. Body temperature variation limits of some farm animals

Animal breed	Average rectal temperature (°C)	Temperature lower limit (°C)	Temperature upper limit (°C)
Beef cattle	38.3	36.7	39.1
Dairy cattle	38.6	38.0	39.1
Buffalo	38.0	37.3	38.6
Sheep	39.1	38.3	39.9
Goat	39.1	38.5	39.7
Chicken	41.7	40.6	43.0
Duck	42.0	41.0	43.0
Goose	40.5	40.0	41.0
Turkey	40.5	40.0	41.0

As in other living beings, all reactions of the body in farm animals are largely dependent on the temperature of the environment, and all cell yeasts acting as catalysts are conditioned to keep the body temperature stable between 38-41.7 °C with a tolerance of ± 0.5 °C. When the body temperature is higher than the

ambient air temperature and the inner surface temperature of the surrounding structural elements (winter-transition seasons), there is a continuous heat loss from the body to the external environment, and in the opposite conditions (summer), there is a continuous heat load from the external environment to the body (Mutaf, 2012)

3. HEAT STRESS IN FARM ANIMALS

Temperature is the most important environmental condition affecting the physiological activities of farm animals. Therefore, temperature is the most important factor to focus on among environmental conditions. Because temperature is a measure of whether the health and comfort of animals are ensured (Borghese, 2005).

The temperature range in which farm animals can perform their productive functions in the best way and feel most comfortable is defined as the ‘‘Comfort Zone’’ and covers a narrow temperature range. The temperatures within the comfort zone are optimum temperatures for animals. Many farm animals are not under thermal stress at average temperatures between 10-20 ℃. Animals consume the least feed and produce the most in the comfort zone (Okuroglu and Delibas, 1986). As the comfort zone is moved away, cold stress sets in at low temperatures and heat stress sets in at high temperatures. Both stress conditions can negatively affect the animal’s meat, milk and egg production.

The seasonal effect of heat stress Is evident In hot and humid regions and especially in the hot summer months. Heat stress can occur with both high and low temperatures. However, since high temperatures are more problematic, when heat stress is mentioned, the problems that occur with high temperatures generally come to mind. There are a number of measures that can be taken to reduce the negative effects of heat stress on health and performance, both administratively and in terms of ration adjustments. The effects of heat stress on animals and their performance are well defined. Rectal temperature increases with high environmental temperature, feed and energy consumption and milk yield decrease. When the ambient temperature exceeds normal limits (5-25 °C) and the relative humidity increases, the thermoregulation ability of the animal Is negatively affected. High-yielding animals are more affected by heat stress than low-yielding ones. Because high-yielding animals are more metabolically active and extra heat loading is higher in these animals. In order

to help animals cope with heat stress, some administrative arrangements such as shading, water misting, shower application, and fan misting should be made. Positive responses can be obtained from these applications in the shortest time. These applications prevent the animal from receiving heat load from the environment and contribute to the evaporative heat removal of the animal from its body (Gorgulu, 2013).

Heat stress, as previously stated, occurs when animals move away from the thermoneutral zone where they have optimum productivity. For this reason, high heat stress is mentioned at temperatures above normal, and cold stress at temperatures below normal. Hypothermia begins to develop in animals at temperatures below normal. Hypothermia is defined as the animal's body temperature falling below normal values. When body temperature drops to between 30-32 °C, there is mild hypothermia. When body temperature is between 22-29 °C, there is moderate hypothermia, and when it is below 10 °C, there is very severe hypothermia. When rectal temperature drops below 28 °C, cattle cannot return to normal body temperature unless external heating or hot water treatment is applied. The environmental temperature that will cause hypothermia varies according to factors such as skin thickness, hair cover, wind speed, and wetness. When hypothermia develops, metabolic and physiological activities slow down. Blood circulation is directed from surrounding tissues to vital organs to protect vital organs. In these cases, the teats and testicles begin to be damaged by frost. If hypothermia progresses, the respiratory rate, heart rate, and blood pressure decrease. The animal loses consciousness and if it is not rewarmed, death occurs in a short time. Animals generally consume more feed to recover from hypothermia. Water consumption also decreases because they need more energy to maintain normal body temperature. In cold conditions, energy requirements can be 25-30% higher than normal maintenance requirements (Gorgulu, 2013).

The amount of heat and water vapor produced by animals inside the shelter varies depending on the temperature and humidity in the indoor environment. For this reason, when the thermal environmental control inside the shelter is not sufficient, the effective utilization of genotypic potential decreases as a result of the negative effect of heat stress and causes productivity losses (Mutaf et al., 2004).

One of the important environmental conditions in animal husbandry is relative humidity. The effect of relative humidity, which varies within certain limits at certain temperatures, on animals is related to the ambient temperature.

Therefore, the relative humidity must also be optimum within the optimum temperature limits. Generally, high temperature and relative humidity cause loss of appetite in animals and reduce the consumption of feed required for maximum productivity by animals. High temperature and relative humidity reduce productivity by reducing appetite and also make it easier for animals to catch diseases (Noton, 1982).

Under optimum conditions for cattle, the relative humidity value should be between 60-80%. The relative humidity value should never be less than 30% and more than 90%. Being below or above the specified values negatively affects the thermoregulatory ability of the animals and as a result, the health of the animal deteriorates (Wathes et al., 1983).

4. CALCULATION OF SURFACE AREA IN FARM ANIMALS

As a general rule, the sensible heat or total heat emitted by the animals to the shelter environment is taken as basis for ensuring heat balance in animal shelters. Unless absolutely necessary for economic reasons, additional heat should not be used.

Animals produce heat by converting the chemical energy of feed into work energy. Animals convert approximately 25-40% of the total feed energy they consume into heat (Ekmekyapar, 1991). The amount of heat and water vapor they emit to the shelter environment is determined either by live weight or by the surface area of the animal. In order to find the amount of heat and water vapor that farm animals emit to the shelter environment depending on their surface area, the surface area of the animals must first be calculated. For this, the values in equation and Table 2 given by Ekmekyapar (1991) and Napolitano et al. (2005) can be used.

$$A = k \times W^{a} \qquad (1)$$

In the equation;

A = Surface area of the animal (m^2, cm^2); k, a = A coefficient; W = Live weight of the animal (kg, g).

Table 2. Relationship between body surface area and body weight of various animals

Animal type	k	a	W	A
Dairy cattle	0.15	0.56	kg	m^2
Beef cattle, Buffalo	0.124	0.60	kg	m^2
Sheep	0.09	0.667	kg	m^2
Chicken	9.85	0.667	g	cm^2

In farm animals, body surface area varies depending on mass; as the living mass increases, the body surface area per unit living mass decreases or the living mass per unit surface area increases.

5. HEAT AND WATER VAPOR EFFICIENCY IN FARM ANIMALS

Generally, when heat-humidity balance calculations are made in animal production structures, the total heat, sensible heat, latent heat and water vapor amounts that animals emit to the shelter environment are taken as basis. Heating is not done unless absolutely necessary. If there is a heat deficit, the insulation of the structural elements is primarily used. On the other hand, when designing ventilation systems in shelters, the minimum and maximum capacity of the system is tried to be determined based on sensible heat for summer conditions, and latent heat or water vapor amounts for winter and transition seasons (Kocaman et al. 2007). In particular, the selection of project criteria regarding the temperature and relative humidity of the region and the determination of their effects on structural features are of great importance. The aim in determining the project criteria should be to provide an economically appropriate balance between the inside and outside of the shelter that will not create heat and cold stress in the animals housed.

a. Total heat production in farm animals

The amount of heat and humidity emitted by farm animals varies depending on the temperature-humidity values of the environment, the sheltering, feeding and watering systems, and varies depending on the animal and shelter levels. The heat and especially the humidity values emitted at the base level of the shelter vary depending on the feeding, watering and sheltering systems, and are higher than the values at the animal level. The heat and humidity values emitted by animals at the base level of the shelter are 20% higher during the day than the average daily values emitted at their own level, and 20% lower than the values emitted at night. (Pedersen, 2002).

Heat-humidity balance calculations for the control of the climatic environment in animal shelters are generally based on the total heat, sensible heat, latent heat and water vapor values given to the shelter environment by the animals. These values are calculated with the help of the equations suggested for farm animals by CIGR (2002), Pedersen (2002) and Mutaf (2012) and given below.

- **Dairy cows**

Total amount of heat (For ambient temperature 20 ℃)

$$q_T = (5.6 \times m^{0.75} + 22 \times Y_1 + 1.6 \times 10^{-5} \times P^3) \times 0.86 \quad (2)$$

- **Horses**

Total amount of heat (For ambient temperature 20 ℃)

$$q_T = 6.1 \times m^{0.75} \quad (3)$$

- **Sheep**

Total amount of heat (For ambient temperature 20 ℃)

$$q_T = 6.4 \times m^{0.75} + 33 \times Y_1 + 2.4 \times 10^{-5} \times P^3 \quad (4)$$

- **Goats**

Total amount of heat (For ambient temperature 20 ℃)

$$q_T = 5.5 \times m^{0.75} + 13 \times Y_1 \quad (5)$$

- **Broiler chickens**

Total amount of heat (For ambient temperature 20 ℃)

$$q_T = 10 \times m^{0.75} \quad (6)$$

- **Egg laying hens (floor feeding)**

Total amount of heat (For ambient temperature 20 ℃)

$$q_T = 6.8 \times m^{0.75} + 25 \times Y_2 \quad (7)$$

- **Egg laying hens (cage feeding)**

Total amount of heat (For ambient temperature 20 ℃)

$$q_T = 6.28 \times m^{0.75} + 25 \times Y_2 \quad (8)$$

The methods for calculating the total amount of heat that farm animals will emit at 20 ℃ ambient temperature, which is considered the appropriate

temperature limits, are given above. However, when the ambient temperature falls below the appropriate temperature limits, the total heat production increases, and when it rises above the appropriate temperature limits, the total heat production decreases. The total amount of heat emitted by animals should be corrected depending on the ambient temperature.

For conditions where the ambient dry bulb temperature is 20 °C below or above, the corrected total heat radiated by animals is:

$$q_{Tcor.} = q_T \times t_{cor.\ fac..} \quad (9)$$

- **For cattle**

Correction factor

When the dry bulb temperature of the environment is between 0 ℃ and 30 ℃;

$$t_{cor.\ fac..} = [1+4 \times 10^{-3} (20 - t_{kt})] \quad (10)$$

When the dry bulb temperature of the environment is above 30 ℃;

$$t_{cor.\ fac..} = [1+3 \times 10^{-3} (20 - t_{kt})] \quad (11)$$

When the dry bulb temperature of the environment is below 0 ℃;

$$t_{cor.\ fac.} = [1+8 \times 10^{-3} (20 - t_{kt})] \quad (12)$$

- **For chickens**

Correction factor

When the dry bulb temperature of the environment is between 0 ℃ and 30 ℃;

$$t_{cor.fac.} = [1+2 \times 10^{-2} (20 - t_{kt})] \quad (13)$$

When the dry bulb temperature of the environment is above 30 ℃;

$$t_{cor.fac.} = [1+1.5 \times 10^{-2} (20 - t_{kt})] \quad (14)$$

When the dry bulb temperature of the environment is below 0 ℃;

$$t_{cor.fac..} = [1+2.8 \times 10^{-2} (20 - t_{kt})] \quad (15)$$

b. Sensible and latent heat ratios in the total heat emitted by farm animals

The total heat emitted by farm animals is the sum of the sensible heat emitted by conduction, convection and radiation, and the latent heat emitted by sweating or evaporation, and can be shown as follows (Ekmekyapar, 1991; Mutaf, 2012).

$$q_{Tcor.} = q_{sen.} + q_{lat.} \quad (16)$$

The ratio of sensible and latent heat to total heat varies depending on the animal's species, breed, productivity, body surface area, hair cover, sweating ability and wetness of the body surface.

The amount of sensible heat emitted by farm animals varies according to the difference between the animal's body surface or body temperature and the ambient dry bulb temperature. These values can be calculated using the equations given below, suggested by CIGR (2002), Pedersen (2002) and Mutaf (2012) for farm animals.

- **For cattle**

Sensible heat amount

$$q_{sen.} = q_{Tcor.} \times [(0.71 \times t_{cor.fac.}) - (0.407 \times 10^{-3} \times t_{kt}^2)] \quad (17)$$

- **For broiler chickens**

Sensible heat amount

$$q_{sen.} = q_{Tcor.} \times [(0.61 \times t_{cor.fac.}) - (2.28 \times 10^{-4} \times t_{kt}^2)] \quad (18)$$

- **Egg laying hens (floor feeding)**

Sensible heat amount

$$q_{cor} = q_{Tcor.} \times [(0.64 \times t_{cor.fac.}) - (2.4 \times 10^{-4} \times t_{kt}^2)] \quad (19)$$

- **Egg laying hens (cage feeding)**

Sensible heat amount

$$q_{sen.} = q_{Tcor.} \times [(0.67 \times t_{cor.fac.}) - (9.8 \times 10^{-11} \times t_{kt}^{6})] \quad (20)$$

The latent heat emitted by farm animals is released as humidity, and its amount increases with the increase in the dry bulb temperature of the environment and the decrease in the humidity of the environment. The amount of latent heat can be calculated with the help of the equation given below.

Amount of latent heat

$$q_{lat.} = q_{Tcor.} - q_{sen.} \quad (21)$$

According to Mutaf (2004), the amount of water vapor emitted by farm animals can be calculated as follows.

Amount of water vapor

$$W_{animal.} = q_{lat.} / 0.680 \quad (22)$$

In the equations; q_T=total heat amount (W), m=live weight (kg), Y_1=daily milk yield (kg/day), Y_2=daily egg yield (0.05 kg/day in production flocks, 0.04 kg/day in incubation flocks) P=gestation period (days), t_{kt}=dry bulb temperature (°C), $q_{Tcor.}$=corrected total heat amount (W), $t_{cor.fac.}$=correction factor, $q_{sen.}$=sensible heat amount (W), $q_{lat.}$=latent heat amount (W), W_{animal}=the amount of water vapor emitted by animals by sweating and respiration (g/h), 0.680=heat of evaporation of water (W).

The equations given above can be used to calculate the amount of heat and humidity that animals emit to the environment. In addition, the values suggested by different researchers in the form of tables below can be used depending on the live weight or surface area of farm animals. For this purpose, Olgun (2012) gives the amount of heat that animals emit to the environment for 500 kg live weight depending on their age, productivity and environmental temperature.

Table 3. Amounts of heat and water vapor emitted by some farm animals to the shelter environment under optimum conditions (Olgun, 2012)

Animal and live weight (kg)	Winter season			Summer season		
	Water Vapor (g/h)	Sensible heat (Kcal/h)	Latent heat (Kcal/h)	Water Vapor (g/h)	Sensible heat (Kcal/h)	Latent heat (Kcal/h)
Dairy cattle						
500	340	666	198	920	262	538
600	370	727	215	1000	284	584
Beef Cattle						
300	225	439	128	605	172	353
500	330	649	194	895	254	524
Sheep						
40	38	72	23	99	28	58
60	40	80	23	110	31	64
Goat						
40	54	107	31	145	41	84
60	65	125	38	175	50	100
Egg laying hen						
1.5	4.4	5.6	2.6	9.0	2.6	5.2
2.0	5.4	7.0	3.1	11.2	3.2	6.5

Ekmekyapar (1991) reported that it would be more appropriate to calculate the amount of heat emitted by the animal to the environment per unit time for each m^2 of surface area depending on the ambient temperature, rather than for each kg of the animal's live weight. The amounts of heat and water vapor emitted by various animals to the environment depending on the ambient temperature and surface area are given in Table 4 and Table 5 (Ekmekyapar 1991).

Table 4. Amounts of heat and water vapor emitted by dairy cattle in closed barns

Ambient temperature (°C)	Latent heat (Kcal/m^2 h)	Sensible heat (Kcal/m^2 h)	Total heat (Kcal/m^2 h)	Water vapor (g/m^2 h)
-6.7	40	175	215	65
-1.1	45	160	205	75
4.4	50	145	195	90
10.0	60	125	185	100
15.6	75	105	180	125
21.1	80	95	175	130
26.7	105	55	160	180

Table 5. Amounts of heat and water vapor emitted to the environment by various sheep breeds.

Koyun ırkı	Fleece cover thick. (cm)	Ambient temper. (°C)	Latent heat (Kcal/m^2 h)	Sensible heat (Kcal/m^2 h)	Total heat (Kcal/m^2 h)	Water vapor (g/m^2 h)
Half.xDown Cross	10.0	13	20.0	36.0	56.0	34.5
Half.xDown Cross	2.5	13	12.5	48.0	60.5	21.6
Half.xDown Cross	10.0	10	16.5	38.0	54.5	28.5
Cheviot	Sheared	8	7.5	82.5	90.0	12.9
Cheviot	3.4-3.8	8	8.6	38.6	47.2	14.8
Cheviot	7.5	12	13.0	39.0	52.0	22.4
Cheviot	10.0	15	24.0	37.0	61.0	41.4
Blackface	Sheared	8	8.6	91.4	100.0	14.8
Blackface	5.8-6.0	8	9.7	37.0	46.7	16.7

Heat balance calculations also have an important place in the design of chicken coops. In the calculations to be made for this purpose, the values given in Table 6 by Ekmekyapar (1991) can be used. The values given in Table 6 are for each kg of live weight depending on the ambient temperature.

Table 6. Amounts of heat and water vapor emitted by some chicken breeds

Chicken breed	Ambient temper. (℃)	Latent heat (Kcal/h kg)	Sensible heat (Kcal/h kg)	Total heat (Kcal/h kg)	Water vapor (g/h kg)
Legorn	-3.3	0.9	6.1	7.0	1.5
	1.7	1.3	4.7	6.0	2.2
	8.3	1.4	4.1	5.5	2.3
	12.2	1.8	3.8	5.6	3.2
	17.2	2.0	3.8	5.8	3.4
	22.7	2.0	3.6	5.6	3.4
	27.8	2.4	3.3	5.7	4.1
	33.3	3.0	0.0	3.0	5.1
Rodey land	4.4	1.0	3.3	4.3	1.7
	10.0	1.2	3.1	4.3	2.1
	15.6	1.2	2.8	4.0	2.1
	21.1	1.3	2.3	3.6	2.2
	26.7	1.3	1.8	3.1	2.2
	32.2	1.6	0.6	2.2	2.8

In order to keep the climatic environment in animal shelters within the desired limits, in addition to structural details, heat losses from building elements and ventilation must be kept at low levels. As a general rule, the sensible heat or total heat emitted by animals to the shelter environment is taken as basis in providing heat balance. Additional heat should not be used unless it is absolutely necessary for economic reasons.

Heat loss and gain in shelters vary according to the seasons. If the necessary precautions are not taken in any shelter, there will be heat gain in the summer and heat loss in the winter. The heat balancing process differs according to these two seasons. In animal shelters, excess heat must be removed in the summer and heat loss must be reduced in the winter (Olgun, 2012).

6. CONCLUSION

In order to regulate the environmental conditions inside animal shelters, it is necessary to know the amount of heat and water vapor that animals emit to the environment inside the shelter. In this study, the equations, principles and data used in calculating the amount of heat and water vapor emitted by farm animals of various breeds and races were brought together with literature research.

As is known, increasing animal production is only possible by housing and feeding animals with improved genetic structure in suitable environments. Otherwise, no matter how good the genetic structure is, if the climatic environmental conditions of the environment in which it is housed are not suitable, the desired yield cannot be obtained from the animal and it will cause the animal to lose its productivity significantly over time.

As a result, when building animal shelters, the type of shelter, the material arrangements to be used and the climatic project criteria for the shelters should be determined by taking into account the climate characteristics of the region where it will be built. According to these criteria, appropriate heat and humidity balance calculations should be made that will not put the animal into cold and heat stress, and the necessary ventilation amount should be calculated accordingly.

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The Role of Natural Plant Based Sources in the Pigmentation of Aquarium Fish

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ABSTRACT

In the aquarium fish industry, coloration holds significant importance in terms of both aesthetic appeal and commercial value. Since fish are unable to synthesize pigments such as carotenoids internally, the use of natural products as external sources of these compounds has become increasingly widespread. Natural pigments are predominantly derived from plants and not only enhance coloration but also support the immune system and overall health of fish. Various plant-based products including carrot, red pepper, spirulina, purslane, and marigold are used for this purpose, offering a safer and more environmentally friendly alternative to synthetic pigments. This study summarizes scientific research conducted on different fish species and highlights that natural pigments can exhibit effects comparable to or even superior to those of synthetic counterparts. In this context, the broader use of natural pigment sources in the aquarium fish industry is recommended, and the importance of further research in this field is emphasized. This study focuses on the utilization of plant-derived products as natural pigment sources in the coloration of aquarium fish.

Keywords – Carotenoids, Fish coloriation, natural pigments, ornamental fishes, fish pigmentation

INTRODUCTION

Aquarium fish attract significant attention due to their aesthetic appeal, biological diversity, and ecological roles. The aquarium fish industry encompasses not only hobbyist breeding, but also scientific research, commercial activities, and ecological conservation efforts (Jones et al., 2022). Globally, the production and trade of aquarium fish constitute a major sub-sector of the aquaculture industry, forming a multibillion-dollar market each year. The global ornamental fish trade is estimated to reach an annual volume of approximately 15–20 billion USD, with over 1.5 billion ornamental fish traded worldwide (FAO, 2021). Leading producer countries include China, Singapore, Thailand, and India, while the largest importers are the United States, Japan, and Germany. Among the most in-demand species in the global market are goldfish (*Carassius auratus*), guppies (*Poecilia reticulata*), discus fish (*Symphysodon spp.*), and neon tetras (*Paracheirodon innesi*). These species are widely favored for their visual appeal and resilience (Monticini, 2010).

The physical appearance of aquarium fish, particularly their coloration, is considered one of the most critical criteria in determining their commercial. Therefore, understanding the mechanisms of fish pigmentation and enhancing coloration through natural means are of great importance from both academic and commercial perspectives. The pigmentation of aquarium fish results from a combination of biological and environmental factors. Fish skin contains various types of pigment cells known as chromatophores, which house pigments responsible for different colors. Chromatophores are classified into several types, including melanophores (containing melanin), xanthophores (containing carotenoids), and iridophores (containing guanine) (Fujii, 2000). While pigmentation is genetically determined, it is also significantly influenced by environmental conditions and nutritional factors. Since fish are incapable of synthesizing carotenoid pigments endogenously, they must obtain them through their diet. Thus, dietary pigments play a decisive role in fish coloration (Luo, 2021).

Both synthetic and natural pigment sources are currently used to enhance coloration in fish. Although synthetic pigments offer advantages such as rapid coloration and cost-effectiveness, their long-term use presents several disadvantages due to potential toxic effects and environmental harm. Excessive use of synthetic pigments may negatively impact biological processes in fish and pose health risks to humans when used in fish intended for human consumption (Malabadi et al., 2022). Additionally, the release of synthetic pigments into aquatic ecosystems may lead to environmental pollution and adverse effects on aquatic organisms. Consequently, there has been growing interest in alternative and sustainable pigment sources.

In recent years, natural pigment sources have received increasing attention in the aquarium fish industry. These pigments are predominantly plant-derived and include various bioactive compounds such as carotenoids, flavonoids, anthocyanins, and chlorophylls (Nabi et al., 2023). These compounds not only enhance pigmentation but may also improve the overall health of fish by strengthening their immune systems. Particularly, plant-based products rich in carotenoids such as carrot, red pepper, spirulina, algae, and tomato are frequently used to promote pigmentation in fish (Kumar et al., 2017). These natural compounds accumulate in the skin and scales of fish, resulting in more permanent and healthier coloration.

One of the key advantages of plant-derived pigments for aquarium fish is their ability to reduce oxidative stress through their antioxidant

properties. Oxidative stress can weaken the immune system of fish and reduce their resistance to diseases. Carotenoids particularly compounds such as lutein, astaxanthin, and beta-carotene mitigate the effects of free radicals, prevent cellular damage, and improve overall fish health (Barad et al., 2024). This provides a significant advantage in commercial aquaculture, where producing healthy and resilient fish is essential.

In addition, the digestion and metabolism of plant-based pigments have positive effects on the growth rate and development of fish. Natural pigments are generally highly digestible, and their bioavailability is often superior to that of synthetic pigments (Ghosh et al., 2022). This further highlights the importance of natural pigment sources for maintaining the long-term health of aquarium fish. Moreover, the natural origin of plant pigments is also a crucial factor in terms of environmental sustainability. While synthetic pigments pose ecological risks due to their chemical content and potential release into aquatic environments, plant-based pigments tend to biodegrade more rapidly in nature (Nambela et al., 2025).

In this context, the use of plant-derived pigments as a primary source of pigmentation for aquarium fish is gaining increasing importance. These natural alternatives are valuable not only in commercial fish farming but also for individual hobbyists involved in aquarium keeping. Their health benefits and eco-friendly nature compared to synthetic pigments support their growing adoption. Nevertheless, further scientific studies are needed to fully understand the efficacy of natural pigments in aquarium fish.

This study aims to examine, from a scientific perspective, the role of plant-derived pigment sources in the coloration process of aquarium fish, their mechanisms of action, and their advantages over synthetic pigments. The promotion of natural pigment use offers significant benefits not only from a commercial standpoint but also in terms of ecological balance and fish health. In this regard, it is essential to encourage new research that addresses existing gaps in the literature. The broader use of plant-based pigments in the aquarium fish industry represents an important step toward more sustainable and healthier aquaculture practices.

In the study conducted by Kıswara et al. (2020), the color change in betta fish (*Betta splendens*) fed with *Artemia salina* enriched with marigold meal (*Tagetes erecta*), which contains high amounts of carotenoids (especially lutein and astaxanthin), was investigated. Among the groups fed with Artemia enriched with mixtures containing different ratios (0, 0.5, 1,

1.5) of marigold meal, the best pigmentation was observed in fish fed with Artemia enriched with a 1:1 mixture of rice flour and marigold meal (1.5). It was determined that this mixture induced a dark red coloration, particularly noticeable in the fin and tail regions of the fish. The study concluded that marigold meal can be used as a natural carotenoid source in ornamental fish farming.

In the study by Jiang et al. (2019), the effects of two different astaxanthin sources natural (*Haematococcus pluvialis*) and synthetic (Carophyll Pink) on the coloration of orchid dottyback (*Pseudochromis fridmani*) were examined. Over a 70-day feeding trial, groups were fed diets containing various concentrations (25, 50, 75, and 100 ppm) of either *Haematococcus pluvialis* or Carophyll Pink. The best results were obtained in the group fed with 100 ppm of *Haematococcus pluvialis*. Compared to synthetic astaxanthin, natural astaxanthin from Haematococcus pluvialis was found to induce faster and more effective pigmentation. It was recommended that natural sources be preferred over synthetic coloring agents in ornamental fish farming.

In a study by Naeem et al. (2021), the potential use of hibiscus leaves, marigold petals, and carrots as carotenoid sources in blue gourami (*Trichogaster trichopterus*) was investigated by adding 15% of each plant to the diet. After a 60-day trial, the highest carotenoid accumulation and pigmentation were observed in the group fed with marigold, while the best growth rate was seen in the control group. The lowest growth and survival rates were observed in the group fed with hibiscus leaves. The study recommended marigold as a low-cost and effective natural carotenoid source for blue gourami.

In the study by Wagde et al. (2018), the use of natural β-carotene sources carrot (*Daucus carota*) and spinach (*Spinacia oleracea*) in the pigmentation of swordtail fish (*Xiphophorus hellerii*) was investigated. Fish were fed for 35 days with diets supplemented with different concentrations (20, 25, 30 mg/100g) of carrot and spinach powders based on β-carotene content. The study found that carrots enhanced red and orange pigmentation, while spinach enhanced yellow and orange hues. The highest red color intensity was observed in the group fed with 30 mg/100g spinach, and the highest yellow intensity in the group fed with 20 mg/100g spinach. The results indicated that natural products such as spinach and carrot are cost-effective and eco-friendly alternatives to expensive synthetic carotenoids.

In the study conducted by Khieokhajonkhet et al. (2023), the effects of three different red pepper extracts (bell pepper, chili spur pepper, and Jinda pepper) on growth, immunity, pigmentation, and disease resistance in goldfish were examined. After 10 weeks, the best results across all parameters were obtained in the group fed with Jinda pepper extract. The extracts significantly enhanced skin pigmentation, particularly in red and yellow hues, and improved resistance against *Aeromonas hydrophila*. The study concluded that red pepper extracts can be used as natural color enhancers and immune boosters in goldfish aquaculture.

The study by Ünver and Hamzaçebi (2020) investigated the effects of natural pigment sources, beetroot (*Beta vulgaris rubra*) and henna (*Lawsonia inermis*) extracts, on the coloration of red zebra cichlid (*Maylandia estherae*). Four diet groups were formed (control, astaxanthin, beetroot, and henna). Although no statistically significant differences were found among groups, an increase in total carotenoid levels was observed in all. The group fed with astaxanthin showed a notable increase in redness, whereas no significant difference was found in lightness (L*) and yellowness (b*) values. The best color stability was recorded in the beetroot-fed group. There were no significant differences among groups in growth performance, feed conversion ratio (FCR), or survival rates. The study showed that natural pigment sources such as beetroot and henna provide comparable coloring effects to expensive synthetic astaxanthin, with beetroot being particularly effective in color stability.

In the study by Kumar et al. (2017), the effects of dietary supplementation of 5% African tulip tree flower, red paprika, and pomegranate peel powders on the coloration of goldfish (*Carassius auratus*) were examined. After a 60-day feeding trial, the highest color enhancement, growth, and survival rates were observed in the group fed with red paprika. The group fed with pomegranate peel showed the highest specific growth rate. The study demonstrated that natural pigment sources can be safely used in goldfish without negatively affecting growth or survival.

The study by Şahin et al. (2021) investigated the effects of purslane (*Portulaca sp*.) extract supplementation on growth and pigmentation in goldfish (*Carassius auratus*). Four diet groups were formed (control (T0), 3% (T3), 6% (T6), and 9% (T9) purslane extract), and the trial lasted 60 days. The best growth performance (0.815 g weight gain, 0.462% specific growth rate) and lowest feed conversion ratio (0.86) were observed in the T9

group. The survival rate was 100% in all groups. The highest color saturation values (Hue (H_{ae}°) angles of 86.73±0.32 and 77.64±0.47) were recorded in the T6 and T9 groups, respectively. The results indicated that purslane extract, especially at high doses (T9), significantly enhanced both growth and pigmentation in goldfish, and improved feed utilization. The study suggested that local, nutritious plants like purslane can serve as economical and sustainable alternatives in aquaculture.

In the study by Joseph et al. (2011), the effects of four ornamental plants (*Hibiscus rosa-sinensis*, *Rosa indica*, *Ixora coccinea*, and *Crossandra infundibuliformis*) added to the diet at different concentrations (1.5%, 2.5%, and 3.5%) on pigmentation and growth in swordtail fish (*Xiphophorus helleri*) were examined. After a 75-day trial, the group fed with Ixora coccinea exhibited the highest carotenoid pigmentation, while the group fed with *Hibiscus rosa-sinensis* showed the highest growth rate. The study reported that the natural carotenoids enhanced orange-red pigmentation and improved the brightness and vibrancy of colors in fish. The authors emphasized the importance of using natural carotenoids in sustainable aquaculture due to their health benefits, natural origin, and lack of environmental impact.

In the study by Sun et al. (2012), the effects of four different pigment sources *Spirulina platensis* (75 g/kg), *Rhodopseudomonas palustris* (200 g/kg), effective microorganisms (200 g/kg), and synthetic carophyll red (1.5 g/kg) on pigmentation and growth in koi fish were examined. After a 99-day trial, Spirulina platensis significantly improved growth, feed utilization, color intensity and brightness in the black and red regions of the fish, as well as the carotenoid and xanthophyll content in the skin and scales. *Rhodopseudomonas palustris* and effective microorganism diets showed no effect on pigmentation. It was concluded that *Spirulina platensis* at 75 g/kg can be used as a natural carotenoid source for koi fish coloration.

The study by Ebeneezar et al. (2020) investigated the effects of dietary oleoresins (paprika, turmeric, and chlorophyll) on skin pigmentation, growth performance, and digestive enzyme activity in clownfish (*Amphiprion ocellaris*), a marine ornamental species. A 60-day feeding trial was conducted with five different diets (control, paprika, turmeric, chlorophyll, and a combination of the three oleoresins). The paprika diet resulted in the highest red and yellow coloration, while the combination diet (COM) provided the highest growth and body weight gain. No significant

differences were found among groups in terms of digestive enzyme activity or body composition. These findings suggested that natural oleoresins, particularly paprika, could serve as effective dietary supplements for improving coloration and growth in clownfish. The study highlighted that natural pigments are safer and more environmentally friendly alternatives to synthetic ones.

In the study by Prabhath et al. (2019), the effects of two pigment sources derived from *Spirulina platensis* (Arthrospira) carotenoids and phycocyanin on the pigmentation of koi carp (*Cyprinus carpio var. koi*) were examined. The 90-day feeding trial involved two color varieties of koi: Kawari (red/orange) and Showa (black and red/orange), and nine different diet groups were established: three phycocyanin diets (PT1, PT2, PT3 with 100, 200, 300 mg/kg), three carotenoid diets (CT1, CT2, CT3 with 10, 20, 30 mg/kg), and three controls (C: basic diet, C+: raw Spirulina biomass, C+1: residual biomass after pigment extraction). The results showed that carotenoid diets, particularly CT3, significantly enhanced red pigmentation in Kawari fish. Phycocyanin diets improved growth rates more than carotenoid diets. Pigmentation was found to be directly correlated with the dietary intake of carotenoids and phycocyanin, although carotenoids had a stronger and more pronounced effect. Residual Spirulina biomass also enhanced both growth and pigmentation. The study demonstrated that natural pigments derived from Spirulina are effective in enhancing koi carp coloration, with phycocyanin promoting better growth.

In the study by Ayi et al. (2018), the use of pumpkin flour as a natural pigment source for koi fish was investigated. Koi were fed commercial diets supplemented with different levels of pumpkin flour (10%, 20%, 30%) over a 40-day period. The best pigmentation was observed in the group fed with 20% pumpkin flour. Pumpkin flour had no significant effect on growth or survival. The study recommended pumpkin flour as a natural pigment source in koi diets.

In the study conducted by Lili et al. (2020), the effects of different levels of marigold meal supplementation (1.0%, 1.5%, 2.0%) to commercial koi diets on coloration, growth, and survival were investigated. The results showed that marigold meal significantly enhanced pigmentation, with the best result observed at 1.5% supplementation. While marigold meal had no effect on survival, it was concluded that it stimulated growth by increasing feed intake. Based on its effects on coloration and growth, marigold meal was recommended as a natural carotenoid source for koi fish.

CONCLUSION

This study comprehensively demonstrates the effects and potential benefits of plant-based products used as natural pigment sources in the coloration of ornamental fish. Coloration, one of the most critical criteria for the aesthetic and commercial value of ornamental fish, is directly related not only to genetic factors but also to environmental conditions and, in particular, to nutrition. Since fish are unable to synthesize carotenoids endogenously, these pigments must be obtained through external sources. In this context, natural pigment sources stand out for their multifaceted advantages over synthetic pigments.

Although synthetic pigments offer rapid and effective results, concerns regarding their potential toxicity, long-term biological effects, and environmental risks have increasingly brought their use into question. Natural pigments, on the other hand, offer an alternative approach in terms of both coloration and fish health. Studies have shown that natural pigments can strengthen the immune system, reduce oxidative stress, and positively influence growth performance.

Experimental studies involving plant-based sources such as marigold, red pepper, carrot, spirulina, purslane, beetroot, and various flower extracts have demonstrated statistically significant effects on fish pigmentation. Among these, spirulina has yielded particularly notable results in terms of both pigmentation and growth. Local and cost-effective plant-based sources like purslane and paprika have also been shown to improve growth and feed utilization rates. These findings highlight that natural pigments contribute not only to aesthetic enhancement but also to healthier fish farming practices. The use of natural pigment sources in ornamental fish coloration holds considerable potential in terms of both fish health and environmental sustainability. Compared to synthetic pigments, plant-based products offer safer, more economical, and environmentally friendly alternatives, while also positively impacting immunity, growth performance, and color quality.

However, it should be noted that these positive effects may vary depending on fish species, pigment source, dosage, and feed formulation. In some studies, although improvements in coloration were observed, no significant differences were found in growth or survival rates. Therefore, the bioavailability and efficacy of pigment sources should be evaluated in detail on a species-specific basis.

Future studies should focus on the standardization of pigment types, determination of optimal dosage levels, and species-specific effects. This will facilitate the integration of natural pigments into commercial feed formulations and contribute to sustainable ornamental aquaculture.

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The Uses Chi Square Tests Family in Fisheries and Aquaculture Studies

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1.Introduction

The fisheries and aquaculture are important topics in the frame of natural source utilization, food security, ecosystem health and conservation. The scope and intensification of the topics are growing in means of research, science, education, technology. By these developments, they are economically growing industries as project implementation, production, sea food proceesing, marketing and employment worldwide.

In every stage of fisheries and aquaculture, statistics and statistical analysis methods are essential needs therefore many statistical approaches and methods are employed in the area. Among them Chi-Square test family frequently used in the statistical analysis of categoric data driven by experiments, field observations and questionnaire studies. The $\chi 2$ test was introduced first by K. Pearson in 1900s, later modifications to the Pearson's $\chi 2$ test was introduced by R.A. Fisher in 1922, by decreasing the degree of freedom by one unit when applied to contingency tables (Bolboaca et. al. 2011, Agresti 2013, McDonald 2014).

In fisheries science, many field observations, measurements and experimental data are collected, if needed transformed and presented as categorically (nominal, ordinal). These data are represented by counting the number of times a particular event or condition occurs. The data analysis, related to associations among data also requires specific methods, the Chi-Square test being one of them (Brown and Guy, 2007, Bolboaca et. al 2011, Agresti 2013).

Many categorical variables have only two levels (success or failure, yes or no), and they are called *binary* variables. Variables having categories without a natural ordering are said to be measured on a nominal scale and are called *nominal* variables. Some variables met to ordered categories. They are measured on an ordinal scale and are called *ordinal* variables. Chi square analyses require assumptions about the random mechanism that generated the data and needs 3 key distributions *binomial*, *multinominal,* and *Poisson* (Agresti 2103, Mc Donald 2014, Das et al. 2023). Some important categorical data in fisheries and aquaculture are; species, predator and prey count, mortality, vitality, sex, catcahability, gear type, gear mesh size, baits, diet composition of a species, consumption preference etc.

Chi-square, a non-parametric test, is an appropriate test when the data are in the form of frequency counts occurring in two or more categories. It enables us to decide on the basis of sample if (1) a given set of counts (or frequencies) statistically match some known, or expected set of values (Goodness of Fit) (2) two or more categories are statistically independent (3) two or more categories are statistically homogenous in case a characteristic (4) is there any other factor that has a confounding effect on independency or homogeinity (Gaur and Gaur 2009, McDonald 2014, Das et al.2023).

Since the basic idea (χ2distribution) and assumptions are identical, original Pearson Chi-square (χ2), Yates' correction in χ2, Fisher's Exact test, Likelihood test, Cochran Mantel-Haenszel test are grouped as Chi-square family test. The test family needs two basic assumptions (a) The data are randomly drawn from a population; (b) The sample size should be sufficiently large. Besides these basics, certain specific assumptions are also needed. χ2 tests are applied for the purpose of goodness-of-fit, homogeneity, independence, confounding factor seeking (Moore and Flinger, 2013, Das et al. 2023). For solving majority of problems in independence testing, Fisher's Exact Test is preferred (Mehta and Patel 2017, Kim, 2017). The Yates' correction for ensuring continuity; Cochran-Mantel-Haenszel procedure (Yıldız and Okyay, 2017) for expressing *another factors'* effect (confounding variable) on relations between two or more categories, could be applied as complementary tests.

Beside some good examples (Neuman and Allen 2007, Bhujel 2008, Qiao 2012, Saputra et al 2020, Luo et al. 2023), the usage the χ2test family is often underutilized or misinterpreted in fisheries and aquaculture research. Additionally, interpretations, effect sizes and direction measurements and post hoc tests are lacked. In classical textbook of statistical methods, biostatistics or biometry, the chi square test applications and examples are accumulated in social science, economics, physicology, medicine and general biology (Zar 2010, Argesti 2013, McDonald 2014, Kim 2017) fisheries topics are rare. The aim of this chapter is to show the possibilities the usage of χ2test family in fisheries, aquatic ecology, aquaculture, fish diseases, fish processing and consumer preferences of seafoods with some examples and conclusions

2. The Chi Square Test Family

2.1. **The Pearson's chi square test**

Pearson's chi square ($\chi 2$) test (simply $\chi 2$ test) is among the most common nonparametric tests. Its properties were first investigated and calculation formula steated by Karl Pearson in 1900. The test is used for data that don't follow the assumptions of parametric tests, especially the assumption of a normal distribution. The test bases on categoric variables (binom, Poisson, multinominal etc.) that have no normal distribution (Argesti, 2013. McDonald 2014).

$$\chi^2 = \sum_{i=1}^{n} \frac{(O_i - E_i)^2}{E_i}$$

where $\chi 2$ Chi square value (calculated, score), O_i: observed, E_i: expected frequencies, *i, j*: categories. In 2x2 and more contingency table the following formula used:

$$\chi^2 = \sum_{i=1}^{r} \sum_{j=1}^{c} \frac{(O_{i,j} - E_{i,j})^2}{E_{i,j}}$$

Where *r* rows and *c* columns in contingency table. The degree of freedom (*df*) is calculated *df=(r-1)x(c-1),* with stated alpha (α,*p*) level and *df* $\chi 2$ value ($\chi 2$ ctitique from the Table 2.3) is compared with $\chi 2$ score.

2.2. Yates' Correction

The application of *X2*test to a small sample could lead to an unacceptable rate of type II error (accepting the null hypothesis when actually false). There is no generally accepted lineation for sample size (minimum sample size varies 20 to 50). Beside the sample size, the counts on cells should be adequate. When no more than 1/5 (20%) of the expected values are smaller than five and there are no cells with zero (0) count. Yates' correction is applied when this assumption is not met (Bolboacea et al. 2011).

$\chi 2$ procedure needs continuity, incase that is not ensured, Yates' continuity correction, as a statistical adjustment, applied to $\chi 2$test for 2$\chi 2$ contingency tables. Its purpose is to prevent the overestimation of significance of $\chi 2$ statistic, particularly when sample sizes are small. The overestimation occurs because $\chi 2$ distribution is continuous, while the data in contingency tables

are discrete (i.e., counts of categories). Yates' correction helps adjust for this difference between continuous and discrete data, and it is an attempt to make the $\chi 2$test more conservative (i.e., less likely to give significant results due to small discrepancies between observed and expected frequencies). When the correction is applying a value of 0.5 must be subtracted from "*the absolute difference*" between observed and expected frequencies. This reduces the magnitude of $\chi 2$, especially when observed and expected frequencies are close, thereby correcting for the tendency of the $\chi 2$test to give overly significant results with small sample sizes.

$$\chi^2_{\text{Yates}} = \sum_{i=1}^{N} \frac{(|O_i - E_i| - 0.5)^2}{E_i}$$

Yates' correction is typically applied in (a) small sample sizes, correction is most useful when the total frequency in the table is less than 20 or when any expected frequency is less than 5 (b) It is primarily used for 2x2 contingency tables. Yates' correction makes the $\chi 2$ test more conservative; however, it has also been criticized for being too conservative, especially when applied to larger sample sizes. Threfore in large samples, Yates' correction may not be necessary. (Bolboacea et al. 2011, McDonald 2014).

2.3. The Fisher's Exact test (FET)

The $\chi 2$ for independence has some difficulties when applied on experimental data. FET was proposed by Fisher as an alternative to the $\chi 2$ test and, is preferred whenever small expected values are presented. FET is based on the calculation of marginal probabilities. FET is used instead of $\chi 2$ when Yates' correction is not acceptable. The most common use of FET is for 2×2 tables. Sample and count size in cells are important and definitive on $\chi 2$test. In case insufficient count (expected frequencies <5 or > 20% of cell count of <5) we need other test applications. FET is more accurate than $\chi 2$ test on small samples, but $\chi 2$ test more accurate than FET on large samples. FET is practically applied in analysis of small samples but it is valid for all sample sizes. While the $\chi 2$ test relies on an approximation, FET is exact one. Especially when more than 20% of cells have expected frequencies <5, it is needed to use FET because applying approximation method is inadequate. FET is applied only in 2x2 contingency tables. FET assesses H_0 hypothesis of independence applying hypergeometric distribution of the counts in contingency table, does not use a mathematical function that estimates the

probability of a value of a test statistic; instead, calculate the probability (*p*) of getting the observed data, and all data sets with more extreme deviations, under the null hypothesis that the proportions are the same (McDonald, 2014). Calculation of ***p*** for FET is done with following formula, in case we have a 2x2 contingency table;

Table 2.1. A sample contingency table for $\chi 2$ test family

	Category B-1	Category B-2	Row Total
Category A-1	a	b	a+b
Category A-2	c	d	c+d
Column Total	a+c	b+d	a+b+c+d = n

p = (a+b)!(c+d)!(a+c)!(b+d)! / (a!b!c!d!n!)

Where; sample size = n, (!): permutation of value; The values (count, number) of sample's categories = a+b, c+d, a+c, b+d Since we need many iterations (repeated calculation) to estimate final ***p*** value by manual computations are time consuming (Mehta & Patel 2013). Therefore, FET is performed basically by statistical softwares.

2.4. Cochran–Mantel–Haenszel Chi Square test

Corchan-Mantel-Haenszel (CMH) procedure was a designed to control of confounding variables when analyzing the association (dependency) between two categorical variables in $\chi 2$test. The idea and of procedure was introduced by Cochran (1954) and later completed by Mantel and Haenszel (1959), therefore somewhere called as Cochran and Mantel-Haenszel separately but generally called as Corchan-Mantel-Haenszel (CMH) test. It's applicable in epidemiology, parasitic fish diesases, aquatic ecology (predator-prey relation, food items and habitat preference) fishing techniques, fishing success, fish gear selectivity, social sciences (marketing, fish consumption, etc.), environmental and biological studies.

The CMH test provides a pooled odds ratio (OR_{MH}) accross the strata of *k* folds, by combining data from multiple 2×2 (or more) contingency tables (strata/groups). It can assess whether there is an overall association between the two variables, while controlling for a third (confounding) variable. In Pearson Chi-square tests, it is assumed that all data comes from the same homogeneous population. If the data is stratified (by site, depth, season, specimen sizes, bait types, life stages or species), it should be adjusted for the stratifying factor by applying CMH procedure. This test is particularly useful in where confounding variables may distort the observed relationship between two categorical variables. Before application the test to verify if homogeneity of odds ratios of strata Breslow-Day test or Tarone's test are applied, if homogeneity ensured ($\chi 2$, df=1, p=0,05 if p>0,05, H_0:is accepted,) then the test is completed (McDonald 2014, 2024 Yıldız and Okyay, 2017).

The variables of CMH test can be classified as (a) *outcome variable* (dependent), a variable of interest for which the researcher want to understand the treatment effect on yield, called as *effect variable*. (b) the treatment variable of interest for which researcher wants to understand the impact of outcome variable, called as *cause* (independent) variable (c) *confounding variable* is an external variable in experiment or observation that effects both both outcome (*effect*) and treatment (*cause*) variables (Figure 2.1). It is expected that counfoundinding variable can cause error or bias in experiment. And it makes confusion and difficulty of the determination the observed of solely effect of the cause variable in the experiment. Therefore, to control of confounding variable's effect is essential to estimate true causal relationship in CMH procedure.

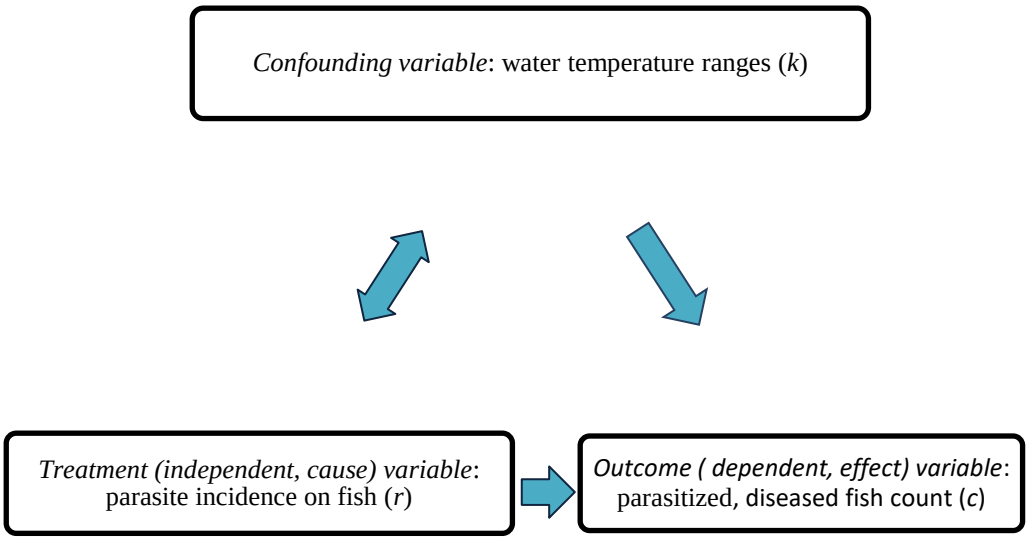


Figure 2.1. The relations of variables in CMH $\chi 2$ test (Table 2.2. Example 4.4.1.)

For CMH estimation Common Odds Ratio (OR_{MH}) is calculated following formula.

$$\mathrm{OR}_{MH} = \frac{\sum_k \frac{a_k d_k}{n_k}}{\sum_k \frac{b_k c_k}{n_k}}$$

Where: a_k, b_k, c_k, d_k are frequencies and $n_k = a_k + b_k + c_k + d_k$ (McDonald, 2014)

OR_{MH} (calculated as 0,1843 in the example data) is an estimate of the odds ratio between two variables **adjusted** for the confounding variable. If $\mathbf{OR}_{MH} = \mathbf{1}$ implies **no association** between *treatment* (exposure) and *outcome* (effect) variables after adjusting for the confounder. When $\mathbf{OR}_{MH} < 1$) suggests a **protective effect** (i.e., the exposure is associated with reduced odds of the outcome). $\mathbf{OR}_{MH} > 1$ implies increased odds of the outcome with the exposure (Table 2.2.).

Table 2.2. The result of new aqua-drug exposure experiment (based on hypotethic data)

Temp. ranges	Exposure	Diseased	Not diseased	Sub-total	Total(nk)
A (10-15°C)	Exposed to drug	*8 a_k*	*24 b_k*	*32*	*61*
	Not exposed to drug	*15 c_k*	*14 d_k*	*29*	
B (16-21°C)	Exposed to drug	10	17	*27*	*52*
	Not exposed to drug	22	3	*25*	
Total	Exposed to drug	*18*	*41*	*59*	*113*
	Not exposed to drug	*37*	*17*	*54*	

To test if there's a **significant association** (H_0: no association across strata), we use:

$$\chi^2_{MH} = \frac{[\sum_k (a_k - E(a_k))]^2}{\sum_k \mathrm{Var}(a_k)}$$

$E(a_k)$ the expected value and $Var(a_k)$ the variance of a_k for 2×2 contingency tables the expected value $E(a_k)$ is calculated as follows:

$$E(ak) = \frac{(ak + bk) * (ak + ck)}{nk}$$

Where;

a_k diseased in group A where exposed to drug (10-15°C) b_k not diseased in group A

c_k disesased in group A where unexposed to drug (10-15°C) d_k not diseased in group A

n_k total sample size in stratum $(a_k+b_k+c_k+d_k)$, Variance Var(a_k)

$$Var(a_k) = \frac{(a_k + b_k)(c_k + d_k)(a_k + c_k)(b_k + d_k)}{n_k^2(n_k - 1)}$$

Mantel-Haenszel procedure, combines the tables (2×2 table) and compares just 2 categorical variables, tests one association. No dependendent to strata number, so *df* in overall test is always 1 (*df=(r-1)x (c-1)* =(2-1)x(2-1)=1 To perform CMH test the homogeinty of starata (*k*) should be assured by Breslow-Day test (p>0,05).

2.5. The application aims of $\chi 2$ *tests*

The $\chi 2$ test family are generally used in a) goodness-of-fit, b) independence (association), c) homogeneity (heterogeinity, variance equality of sample and population) d) Seeking confounding factors/variables e) classification of categories etc. (McDonald, 2014, Zar, 2010, Bolboaca et al., 2011, Das et al. 2023).

2.5.1. Goodness-of-fit (GoF) test

The $\chi 2$GoF test may be called as "One Sample $\chi 2$". GoF means how well a sample of observations from a population of data conforms to the population's distribution of observations expressed by a H_0 hypothesis. GoF procedure compare frequencies in a sample to frequencies hypothesized in the sampled population. In testing such hypotheses, $\chi 2$ is widely used. Some other test (such as loglikelihood ratio, Kolmogorov-Smirnov, Shaphiro-Wilk test, Watson test etc.) devoloped and used for GoF. GoF determines if the sample under investigation has been drawn from a population, which follows some specified distribution. The hypotheses of GoF are H_0: The observed distribution of frequencies equals the expected distribution of frequencies in each category. H_1: The observed distributions of frequencies does't equal to the expected distribution of frequencies. When we apply it one special topic, we should convert it the scope of topic. The test statistic is the same Pearson $\chi 2$ square as;

$\chi 2 = \sum_{i=1}^{k} \frac{(O-E)2}{E}$ where $\chi 2$ Chi square value (calculated, score), *O*: observed, *E*: expected frequencies, *k*: category number. To estimate expected frequencies (*E*), the known proportions of each category (such p=0,5 0,3 0,1 0,05 and 0,05 etc.) is taken and multiply by total of obtained frequencies,

observed total or if we assume the equality expectations, we multiply total sum of observed frequencies by expected ratio (*E=(x/1)*Total observed*. Therefore all the expected values will be the same. As a classical example probability of one face of Backgammon dice's is 1/6 therefore total observed frequency will be multiplied by 1/6. And probability of male or female in a fish population equal to 0,5

For $\chi 2$ GoF testing, to estimate $\chi 2$ critique value we need *df* and significance (error) level (α). Degrees of freedom *df=k-1* and (α) is choosen by researcher's opinion or research topic neccesities; in biological surveys and trials generally choosen as α=0,05 if need (or detailed investigate the issue) as α =0,01 or α =0,10 (mainly for social and economic issuses, higher subjectivity may be expected). Then two $\chi 2$ is compared, if $\chi 2_{\text{score}} < \chi 2_{\text{critique}}$ H_o accepted (observed distribution of frequencies equals to the expected); If $\chi 2_{\text{score}} > \chi 2_{\text{critique}}$ H_0 rejected (observed distribution of frequencies does't equal to the expected). Critical $\chi 2_{\text{critique}}$ are given by significance level (α) and *df* in Table 2.3. .

2.5.2. Chi-square independence test

In some studies, we meet investigations of frequencies of statistical associations of two categorical characters. The $\chi 2$statistic can be used when two or more categories are involved for two (or more) attributes. *X2*test of independence is used to test the hypothesis that two categorical variables are independent of each other. A small $\chi 2$statistic indicates that H_0 is correct and that the two variables are independent of each other. *X2* test of independence is named also as "association $\chi 2$ test. The $\chi 2$ test of independence is applied in order to compare frequencies of nominal or ordinal data for a single population/sample (two variables at the same time). (Das et al. 2023)

The procedure involves comparing the observed cell frequencies with the expected cell frequencies. Observed cell frequencies are the actual number of cases falling in different cells of the contingency table and expected frequencies are the number of cases that should fall in each cell if there is no relationship between the two categorical variables. The basis of the test is difference between the observed frequency and the expected frequency of each cell of the contingency table. Observed cell frequencies can be directly obtained from the given data, the expected cell frequencies are calculated by

multiplying the total of the row by the total of the column to which the cell belongs and then dividing by the total sample size.

$\chi 2$independence test has some difficulties when applied on experimental data. Whenever small expected values are estimated Fisher exact test was proposed as an alternative to the $\chi 2$ test. FET is based on the calculation of marginal probabilities (which unfortunately has an exact calculation formula only for 2×2 contingencies). H_0: Observed distribution of frequencies equal the expected distribution of frequencies in each category. H_1: observed distribution of frequencies not equal the expected distribution of frequencies. Those are statisticaly constructed hypothesis, when we apply it one special topic, we should convert it the scope of topic. It is useful to look at contingency tables and results of $\chi 2$test to gain meaningfull insight into the data. Contingency tables are present the data in rxc tables (2x2, 2x3, 3x3, 3x4 etc.).

O_{ij} and, E_{ij} = 1, 2, . . ., r; j= 1, 2, . . ., c refers to observed frequency (the data) and refers to the expected frequency (based on the hypothesis) corresponding to i^{th} row and j^{th} column of the rxc contingency table, then $\chi 2$ statistic is calculated by;

$$\chi^2 = \sum_{j=0}^{c} \sum_{i=1}^{r} \frac{(O_{ij} - E_{ij})^2}{E_{ij}} \sim \chi^2_{(r-1)(c-1)}$$

The expected frequency of each cell is determined by $E_{ij}=A_i*B_j/N$ where A_i *sum of related row* , *Bj* sum of related column N total number of frequencies in obserrvations/or trial (general total for all categories and variables)

After calculation of $\chi 2$statistic, we need degrees of freedom (*df*) associated with the contingency table to find the significance of the relationship (*df=(r-1)x(c-1)*) (r, c row and column numbers). The *df* and significance level (α) are used to find the values of $\chi 2$ critique from the tables (Table 2.3.). If the tabulated $\chi 2$value is less than the calculated $\chi 2$value, the H_0 is rejected and we conclude that there is some significant association between the two variables. The test can also be applied to ordinal categorical variables. While

we can test the independence of relationship between only two variables at a time, the variables can have any number of levels.

2.5.3. Chi-square homogeneity test

The $\chi 2$homogeneity test investigates whether several populations (or the same population in different years, seasons, places etc.) are homogeneous with respect to a particular characteristic. In homogeinity tests; (a) it is aimed to analyze if different populations are similar (homogenous or equal) in terms of some characteristics (ie. Size and age structure of a population, stock, catch etc.). The $\chi 2$ homogeneity test is used to determine whether frequency counts are identically distributed across different populations or across different sub-groups of the same population. (b) to verify the homogeneity of data, proportions, variances (ie. equality of variances).

An important assumption for homogeneity test is that populations come from a contingency of two or more categories (Neuman and Allen 2007, Bolboacă et al. 2011, Das et al. 2023). By using observed values (counts) contingency table is constructed; the values of first category (factor, population, subgroup, seasons etc.) are put in the rows and the values second category (factor, population and subgroup) in the columns. The *df* is calculated as in independence $\chi 2$ α is choosen by researcher's opinion or the topic's neccesities, in fisheries surveys and aquaculture trials generally choosen as α=0,05. Hypoteses are constructed as H_0: The populations are homogenous in certain characteristic (eg. fish length distribution) H_1:the populations are not homogenous (the studied character has different distribution). The test procedure is the same as $\chi 2$ independency test.

2.5.4. Confounding Factor Seeking

In some research we need to express the other factor's effect on the relation. For these cases a special $\chi 2$ test that modified by Cochran-Mantel-Haenszel (CMH) used to assess conditional independence while controlling for confounding factors. (See sec. 2.4.)

2.6. Assumptions for Chi-square tests

The basic assumptions are (a) The data are must randomly drawn from a population (b) Each observation must fall in one and only one category (i.e. the observations in the sample should are independent of one another) (c)

The observations are measured as frequencies, when given as percentages they must be convert to fequencies. (d) The sample size is should be sufficiently large. The expected frequency for each category must not be less than 5 for *df* = 2 and not less than 10. For *df*=1, the "observed-expected" must be corrected for continuity (Yates' correction) in order to use the table of values of critical. In experiments and field observations sample size can be independent to the researcher; therefore there are no accepted limits for the sample sizes. But in general, accepted minimum sample size varies from 20 to 50. The values on cells in RxC tables are adequate when no more than 1/5 of the expected values (>20%) are smaller than 5 and there are no cells has zero (0) frequency. (Bolboacă et. al. 2011, McDonald 2014, Das et al.2023)

2.7. Hypothesis building in χ2 test family

Hypothesis building differs with the test aims (goodness of fit, independence, homogeneity, variance equality, confounding factor analysis).

a) **Goodness of fit tests** whether a sample distribution fits a specified population distribution, H_0:the sample distribution matches the expected distribution (the values/percentages etc. ***fit good*** to therotical/known distributions, normal, binom, Poisson, values, percentages etc.) H_1: The sample distribution differs from the expected distribution (the present values ***not fit good*** to therotical/known distributions).

b) **Independence tests** that determines if two categorical variables are related, H_0: the categorical variables are ***independent*** (no association); H_1: variables are ***not independent*** (dependent or have association with eachother).

c) **Homogeinity test** that compares distributions across multiple groups, H_0: The distributions are the same across groups. H_1: at least one group differs from others. In comparation of the population and the samples' variances, H_0: population and sample variances are ***equal*** H_1: Variance of the sample ***not equal to*** population, population and sample variances differ.

d) **Cochran-Mantel-Haenszel (CMH) test that** designed to assess **conditional independence** while controlling for confounding factors, H_0: the association between the categorical variables remains the same across different strata (time, place, replication etc.) of the confounding variable (e.g. the variables are independent within each stratum). H_1: there is a

significant association between the categorical variables; even after controlling for the confounding factor (e.g. the variables are dependent within at least one stratum).

2.8. χ2 Test procedure

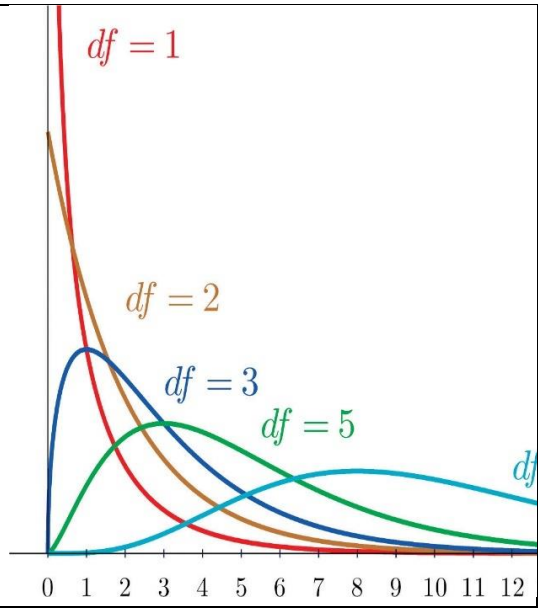
Test procedure follows these steps: i) hypothesis formulation (H_0, H_1 see sec. 2.7) and choosing significance level (α) ii) preparation of contingency table by using data iii) calculating expected frequencies and degree of freedom iv) calculation of $\boldsymbol{\chi 2}$ statistics v) comparing the χ2 score or *p* value and interpreting them vi) performing the appropriate supplementary tests (Phi, Cramer's V, Gamma etc.) and if H_0 rejected, post hoc tests are applied and interpreted their scores. Basic formula of χ2

$\boldsymbol{\chi 2} = \sum_{i=1}^{k} \frac{(O-E)2}{E}$ where χ2 value (statistics, score), O observed, E expected frequency,

Critical values of χ2 (*df*, *α*) are found in table 2.3., statistics text books and internet sources. For χ2 critique value (*df*) is highly influential (Table 2.3.) and calculated as *df= (r-1)*(c-1)*. r: number of rows (category A), c=number of columns (category B) of the contingency table. The significance (error) level (*α*) is choosen by researcher's opinion or research topic neccesities, in biological surveys and trials generally choosen as *α*=0,05. If χ2 score < χ2 critique H_0 hypothesis is accepted; if χ2 score > χ2 critique rejected.

Table 2.3. Critical χ2 values in respons to *df* and *α* levels and *df* effect on χ2 (graph) (https://saylordotorg.github.io/text_introductory-statistics/s15-chi-square-tests)

Critical values for χ^2			
p-val / DF	0.1	0.05	0.01
1	2.706	3.84	6.64
2	4.605	5.99	9.21
3	6.251	7.82	11.35
4	7.779	9.49	13.28
5	9.236	11.07	15.09
6	10.65	12.59	16.81
7	12.02	14.07	18.48
8	13.36	15.51	20.09
9	14.68	16.92	21.67
10	15.99	18.31	23.21



2.9. Strenghts and challenges of $\chi 2$ tests

$\chi 2$ test family have following benefits and strengths: a) The tests are easy to apply with categoric data b) No strict assumptions about data distributions (normal, binomial, Poisson, geometric etc.) c) widely applicable in different scientific researches (biology, aquatic ecology, health etc.). But have some challenges and limitations as a) sufficent sample sizes are needed for accurate and safe results b) the tests are sensitive for expected frequency assumptions c) the tests can not establish causalition, they inform about independency and association.

The followings might be cause errors or misunderstanding of $\chi 2$ test results a) ignoring sample size requirements, minimum counts of cells (<5) and the percentages of under count having cells (>20%) b) misuse the $\chi 2$ test type in small sample studies (assumtions of used test type were not met) c) confusing correlation with causation, a significant $\chi 2$ result suggest only an association between variables, it does not establish a causation d) overlooking assumption violations (test type, sample size, cel count) e) incorrectly grouping of sample data, inproper binning of continuous variables to categorical groups can distort results (in case of lengt, weight, water temperature, dept of habitat). For example, if water temperature is split

into to “cold” and “warm” without the ecological thereshold respect to the species, variability might be lost.

The same distortaion might be met in length frequencies for different sized fish species (which length class range is appropriate? 0,5 cm 1 cm 5 cm or 10cm (see Table 4.1.) e) misinterpreting not significant (p>0,05) result, it does not neccesarily means there is no association, it could be due to insufficient statistical power or small sample size f) Using $\chi 2$ test for quatitative data, the test family are designated for categorical data. Applying it continious variables (length, weight) without proper categorization is not appropriate h) ignoring effect size, statistical significance not enough alone, the effect size (Cramer’s V should be estimated to asses the strength of relationships (softwares suply Cramer’s V value Table 4.3) i) misscalculating or choosing of *df*, erros in *df* calculation can be lead to incorrect test application affecting *p* values and results. It should be noticed $\chi 2$ tests are highly sensitive to *df* (Table 2.3).

2.10. Interpretation of test result and measuring the association

The $\chi 2$ independence test is an overall test for detecting relationships between two categorical variables. If the test result is significant ($p< 0,05$), it is important to look at the data to learn the nature of the relationship by several ways;

a) *Comparing selected percents:* which cells occur in very different percentages than the other cells? b) *Comparing observed and expected cell counts*: which cells have more or less counts than would be expected if H_0 was rejected

c) *Looking at the terms of the $\chi 2$ statistic*: which cells contribute the most to the value of $\chi 2$ (remember $\chi 2$ is a summed one, $\sum$). Threfore researchers can conclude not only that there is an association between the variables, but they can also describe the association.

d) Effect size $\chi 2$ independence test of two nominal variables does not describe the strength of association between them. Therefore, it should be determined the degree of association by estimate *effect size (symmetric measures*). The effect size is determined using phi (φ) coefficient (ranges between 0-1). If phi~ 0.10 effect size small; =0,3 medium and =≥0.50 large.

In 2x2 contingency table phi(φ)=$\sqrt{x2/n}$; as in Table (4.3) (φ)=0,125 means a small effect size on gear type x bait kind.

If the contingency table more than (2x2) Cramer's *V*=$\sqrt{x2/n(L-1)}$ can be used. Here, $\chi 2$ test statistic, *n* count of whole sample, *L* minimum total count of row/column in contingency table (Das et al., 2023). In 2x4 contingency table Cramers's V= 0,125 shows small effect size. In both phi (φ) and Cramer's V value are significant p=0,000 <0,05 (Table 4.3). Phi (φ) and Cramer's V express how strong that association is. Therefore, to decide the size of effect we should use the special scale for the effect size estimation results. In case of Table (4.3) the association was statistically significant ($\chi 2$ =44,151, df= 3, p=0,000), but the effect size was small (φ = 0.125).

2.11. Post-Hoc Test for $\chi 2$

The $\chi 2$test assesses general question whether relation between two variables is independent or associated. To make clear the situation, we need further evaluation to produce final information regarding of issue. If there are three or more levels of variables (2x3, 3x3, 2x4 etc.), a post-hoc pairwise comparison is required to compare the levels of each other. In comparation the prevalence of a certain disease, there are 3 comparative groups (ie. control, treatment 1, treatment 2) and when the $\chi 2$test concludes that there is significant association (dependency), we may want to know if there is any significant difference in three compared pairs (between control and treatment 1, control and treatment 2). In the case, we can reduce the table into multiple 2x2 contingency tables and perform the $\chi 2$ test with applying the Bonferroni corrected alpha level (corrected α = 0.05/3 = 0.017) to compare the pairs.

3. Chi square tests in fisheries and aqaculture

3.1. Where we use $\chi 2$ tests?

The $\chi 2$test family has been applied in all research areas to analyse categorical variable data. Its main uses are goodness-of-fit, association/independence, homogeneity, classification, variance comparation etc. (Neumann and Allen 2007, Bolboaca et. al. 2011, Argesti, 2013, McDonald 2014). The $\chi 2$ test family is used biosciences (biology, botany, zoology, ecology, genetics, medicine, physiology etc.) and social sciences (education, economy, marketing, politics) more than a century. The test is even used in highly quantitative fields such as engineering sciences and industrial quality control (eg. classification of items according to whether

they conform to certain standards, and subjective evaluation of some characteristic, how durable a certain fabric, food is, how good a particular food product tastes, or how easy a worker finds it to perform a certain task).

3.2. Aquatic ecology and fisheries

$\chi 2$ test is used in aquatic ecology and fisheries especially analysing categorical data to test independence or associations between the variables. Biodiversity, aquatic organism distribution between the habitats, habitat quality evaluations, categorization the habitats depending on characteristics (vegetatiton type, substrate, depth) and their suitability for different fish and other animals. The impacts of habitat altrerations on the occurrence and density of animals, to assess the efficiacy of remediation techniques.

The sex ratio and age composition comparations in different lakes, ages, responds to fishing methods, seasons, depts, day times, subsequent years etc. The test are used in population genetics, to determine allel frequencies, genetic variations of qualitative treaths, behavioral studies, to assess of foods and habitat preferences, feeding habits, behaviors the species to environmental factors and relation to them. Impact factors on fish survival and deaths and associations between them. The tests are also used fisheries management, comparing the catch composition to fishing methods, gears, location, baits and seasons. GoF can be assessed by a $\chi 2$test by comparing the observed catches with the expected catches, catchability over time (Brown and Guy 2007, Neumann and Allen 2007, Alaş et al. 2010, Quaggio et al. 2011, Quiao 2012, Saputra et al. 2020, Demirkıran and Özekinci 2022, Das et al. 2023, Luo et al. 2023). The $\chi 2$ tests are used to test the differences in length frequenceies among the samples, their dependency of the treatment (water body, gear type, or time period). $\chi 2$ tests are applied, but not limited to, length-frequency data for which the length-groups are rather large. Length data are often categorized using stock density index length categories rather than by more detailed length intervals (Neumann and Allen 2007). Sapurta et al (2020) examined the lobster species (*Panulirus* sp.) abundance and species composition that live east Jawa coast in manner of habitat depth by using $\chi 2$ test. The tests can be used to define disrubution patterns of fish and other organism abundance in different place, dept.

Ecological niche and interspecific relationships among the main fish species in the coastal waters (Hainan island, China) that taken from two bottom trawl stock surveys conducted in two seasons (May, September), were analysed. Interspecific associations were controlled by $\chi 2$test, based on a 2x2

contingency table and Yates' correction was applied. There were 56 species pairs in the 2 seasons that were significantly ($\chi 2 \geq 3.841$) associated (Luo et al., 2023).

Gear (net, trap, trawl, fyke net, fishing pot, hook type/size) selectivity is vital characteristic in commercial and sport fisheries. Proper selectivity ensures population and fishing sustainability, control and reduce legal/sublegal size of cath. In this context, the data of experiments and field surveys are mainly categoric and they should be analyzed by $\chi 2$ test family to test dependence and association (Qiao, 2012, Demirkıran and Özekinci 2022)

3.3. Aquaculture research and aquatic animal diseases

In aquaculture research, the results/measurements may be categorical or transformed to categorical scales or groups (temperature ranges, feeding ratios, yes/no responses, dead/alive, egg hatching success, survival rates, regions, farms etc.), for such circumstances, $\chi 2$ test family can be used efficiently (Amos et. al. 2018, Dominguez et. al., 2023).

In fish farms to asses the independency of disease incidence, disease prevalence studies and comparing pathogene succeptibilty in particular conditons (water quality, toxic chemicals); to answer the prevalence of certain diseases differ between the stocks, culture techniques, prophylaxy, fish farms, efficacy a chemical drug (formaline, chloramine-T) or medication to cure a parasitic diseases (recovered, partially recovered, not recovered etc.). In fish and aquatic organism genetics, type of allele inherited by an offspring (Lindemann et al. 2011), in aquaculture diet and nutritional studies, to assess the relation between specific nutrition and disease/resistance $\chi 2$ test are used (Bhujel, 2008).

3.4. Seafood processing, marketing and consumption

Analysing market preference for fish species based on consumer data. Frequently, fish consumption habits and preferences in relation to socioeconomic groups, education levels, professions, rural and urban consumers were tested by χ^2 test (Aydın et. al 2011); gender, education levels, income classes, cooking methods and influencing factors were analysed by FET χ^2 (possible independency-association) test based on constructed congintency tables FET were used also to verify possible associations between the consumers' characteristics and fish consumption habits (Can et. al., 2015). Fish consumption preferences in Turkey's costal

regions (Marmara-Black sea, Aegean, Mediterranean) is studied by Sagun and Saygı (2021). Questionaries were evaluated by χ^2 tests.

3.5. The other usages of χ^2

The socioeomomical statue, employment, labor preferences of fishermens and aquaculturist can be assessed in field observations and questionaries. In such research many categoric variable are met. To test them and drawing considerations χ^2 test family used.

4. Application examples of χ^2 tests in fisheries and aqaculture

4.1. Goodnes of Fit (GoF) test

The χ^2 GoF test can be used to test normality, to control the hypotheses that the data wheter comes from a normal distributed population. H_0: data are sampled from a normal distributed population (distibution is normal); H_1: data are not sampled from a normal distributed population (disribution is not normal). The test can be applied two situations a) population parameters are known b) population parameters are not known (estimated from the sample).

4. 1.1. Normality test of length frequency distribution of *G.aculaetus* (Example 4.1.1.)

From the Dalaman river (South-west Anatolia) 83 sticleback (*Gasterosteus aculeatus*) were sampled, their total length measured (cm), the frequency table of length groups constucted (0,5 cm int.), a histogram drawn (Figure 4.1.). The mean (X=3,92 cm)and standard deviation (SD=1,52)were calculated by Excel (since population parameters are not known). *Df* calculated as =(k-1)-2=12-1-2=9 (of parameter numbers (2) subtracted from *df* .

Lengths (cm)	1,25	1,75	2,25	2,75	3,25	3,75	4,25	4,75	5,25	5,75	6,25	6,75
Frequencies	1	7	14	11	5	2	11	3	16	5	7	1

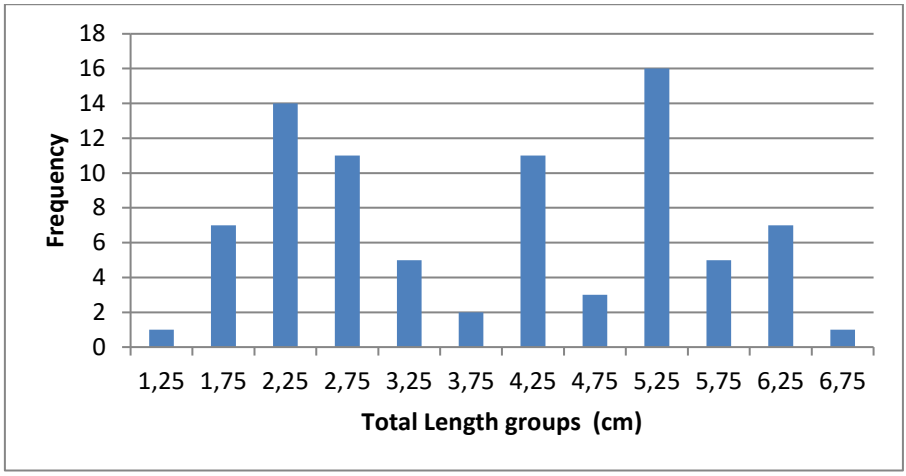


Figure 4.1. Length frequency histogram of Dalaman river *G. aculeatus*

To estimate expected frequencies, possibilities were calculated by using NORM.DIST (ni; x; SD; N) function. ni: frequency of ith category, x: average length of the sample , SD: standard deviation, N: sample size. After multplying obtained possibilities with observed frequencies (O) we obtained expected frequencies (E) (Table 4.1.). To perform χ^2 normality test, we checked expected fish number (E) in each category. Notice in 6 categories (50%) count numbers (yellow cells) are less than 5, so we must use Yates' corection factor ($\chi^2 = \sum(|O - E| - 0{,}5)\text{^}2/E$). It is estimated by excel function CHSQ=(ABS(O-E)-0,5)^2/E) Sum of CHSQ is found as 27,974. CHSQ-critique is found by CHSQ.INV.RT(α; df) as 16,919 (look Table 2.3 also). Sum of CHSQ (χ^2statistic)> CHSQ critique χ^2 Ho: is rejected, the distribution is not normal. In evaluation of *p*, it can be find by excel function CHSQ.DIS.RT (sum of CHSQ;df) as 0,001, the proposed p =0,050 so, 0,001<0,050 H_0: is rejected, the distribution is not normal. If we review the frequecy histogram (Fig. 4.1), it is clearly seen the sample has polymodal distribution (at least 3 mod) possibly due to several age groups are included.

Table 4.1. χ^2GoF test Excel worksheet of *G. aculeatus* sample

L(cm)	FRQ-(O)	POSSIBILTY	EXP-FRQ (E)	CHSQ
1,25	1	0,0276	0,0276	8,0710
1,75	7	0,9787	6,8506	0,0179
2,25	14	1,0000	14,0000	0,0179
2,75	11	1,0000	11,0000	0,0227

3,25	5	0,7619	3,8094	0,1252
3,75	2	0,1040	0,2079	8,0302
4,25	11	1,0000	11,0000	0,0227
4,75	3	0,2736	0,8207	3,4365
5,25	16	1,0000	16,0000	0,0156
5,75	5	0,7619	3,8094	0,1252
6,25	7	0,9787	6,8506	0,0179
6,75	1	0,0276	0,0276	8,0710
			SUM of CHSQ	**27,9738**

4.2. Independence test

4.2.1. χ^2 test for gear x bait types of crayfish catch experiment (Example 4.2.1)

To estimate the efficiency of gear types (fyke net , fishing pot) and bait types (fish, bread, chicken no-bait) and their dependence in crayfish fishing an experiment was performed (Demirkıran and Özekinci, 2022) The obtained data were given in a 2x4 contingency table (Table 4.2).

Table 4.2. Gear types x bait kinds contingency table for crayfish catching experiment (Demirkıran and Özekinci, 2022)

		Bait types				
Gear types		Fish	Bread	Chicken	No-bait	Total
	Fyke net	534	519	568	319	1940
	Fishing pot	224	187	365	104	880
Total		758	706	933	423	2820

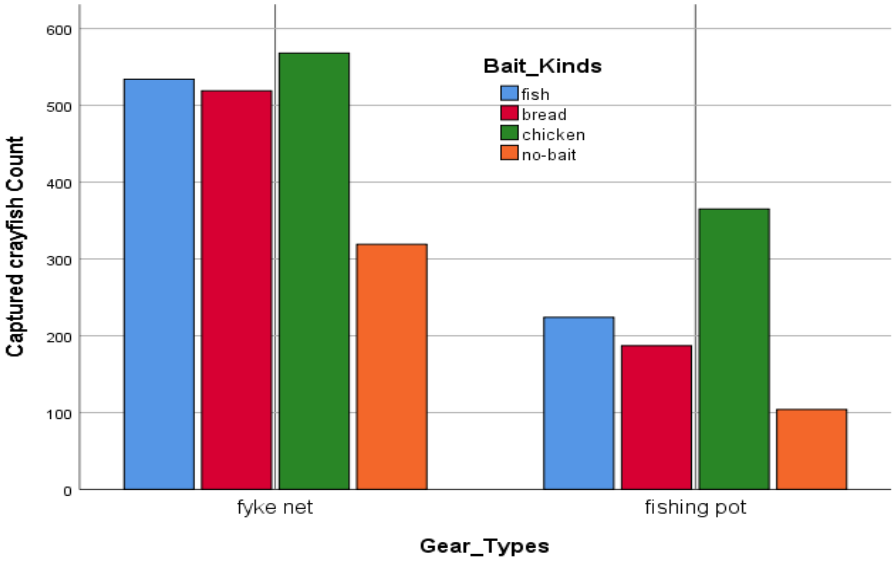


Figure. 4.2. Crayfish counts by gear types and bait kinds in crayfish catching experiment

In SPSS a data file is prepared by determining variables, coding them, and weight the cases (DATA>Weight cases to crayfish counts) Next, ANAYZE>DESCRIPTIVE STATISTIC> CROSS TABBS in this stage, transfer gear types to row and bait types to column boxes, choose display clustered bar cards (if needed). Then click STATISTIC> In crosstabs: In statistics choose Chi Square, Phi and Cramaer's V than click CONTINUE. Click CELLS and there choose expected counts, percentages of rows or columns and Adjusted standard residual CONTINUE > OK. Following outcomes is presented.

Table 4.3. Gear types x bait kinds cross tabulation, χ^2 and effect size test result for crayfish catching experiment

Gear_Types * Bait_Kinds Crosstabulation

			Bait_Kinds				
			fish	bread	chicken	no-bait	Total
Gear_Types	fyke net	Count	534	519	568	319	1940
		Expected Count	521,5	485,7	641,9	291,0	1940,0
		% within Gear_Types	27,5%	26,8%	29,3%	16,4%	100,0%
		Adjusted Residual	1,1	3,1	-6,4	3,2	
	fishing pot	Count	224	187	365	104	880
		Expected Count	236,5	220,3	291,1	132,0	880,0
		% within Gear_Types	25,5%	21,3%	41,5%	11,8%	100,0%
		Adjusted Residual	-1,1	-3,1	6,4	-3,2	
Total		Count	758	706	933	423	2820
		Expected Count	758,0	706,0	933,0	423,0	2820,0
		% within Gear_Types	26,9%	25,0%	33,1%	15,0%	100,0%

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	44,151[a]	3	,000
Likelihood Ratio	43,711	3	,000

a. 0 cells (0,0%) have expected count less than 5. The minimum expected count is 132,00.

Symmetric Measures (size effect)

		Value	Approximate Significance
Nominal by Nominal	Phi	,125	,000

	Cramer's V	,125	,000

Results and evaluation:
χ^2 =44,151, *df*= 3 (2-1)*(4-1) , *p*=0,000<0,05, H_0: rejected. The gear types and bait kinds are not independent; there is some association/dependency between the categories. χ^2 test shows there is to be a significant relation (association) between the categories (<0,05) to perform a post hoc test in SPSS, we return the analyze again and follow:
ANALYZE>DESCRIPTIVE>CROSSTABS>CELLS in the dialog box chose observed, expected and Adjusted standardized then >CONT. >OK. In "CELLS" dialog box the rows or columns percentages can be also choosen to express how much the categories' pies contribute to total (%100). In output, the values relating to tabulated categories can be concluded.

1) In a proximate evaluation, in case of fyke net x bait kind realation highest observed and expected counts (568, 641) and percentage (29,3%) are seen in fyke net-chicken meat association. In case of fishing pot x bait kind, highest observed and expected counts (365, 291,1) and percentage (41,5%) were in fishing pot x chicken meat association.
2) Acording the Adjusted standard residual, fykenet**x**bread; fykenet**x**chicken and fykenet**x**no-bait association and fishingpot**x**bread, fishingpot**x**chicken, fishingpot**x**no-bait that are the out of normal distribution (< or > 1,96) are significant the rest are not significant. The highest value was estimated as -6,4 and +6,4 in case of fyke net**x**chicken and fishing pot**x**chicken asosociations are stronger than others. So chicken meat is an efficient as bait for both fishing gears.
3) For further consideration, χ^2 test for 2x2 contingency tables are constructed and estimation of *p* value is needed in this case *df*=1 and comparation *p* value =*alfa/df* (for total test) here; 0,05/3= 0,017 (Benforroni correction). Separate χ^2 test *p* results can be estimated by excel easily (χ^2=∑(observed counts-expected)^2/expected) and *p*= CHISQ.DIS.RT (χ^2score;*df*). Considering *p*=0,017), fyke net**x**chicken (*p*=0,004) and fishing pot**x**chicken (*p*=0,000) associations are significant, the others are not. In both gear type chicken gives more crayfish catch. In total, chicken meat's catch success (33,1%) is the highest followed by fish (26,8%).
4) To asses the strength of association (size effect, categories nominal, RxC as 2x4) Phi or Cramer's V was used (0,125). The association strength is small but statistically significant (*p*=0,000)

4.2.2. Independence test black sea bass (*C. striata*) experiment (Example 4.2.2.)

On the analysis of black sea bass (*Centropristis striata*) catch counts, "size legality -sublegal and legal" and "vent size cm-getting wieder" are organised as ordinal variables. We want to test the relation with legality and vent size (Qiao, 2012). The test was performed by using SPSS as before explained, Gamma was chosen in statistics dialog box (ordinal variables) to measure the strength of association. The outcomes were as follows:

Table.4.4. Black sea bass *C. striata* experiment cross tabulation, χ^2 and effect size test results

Size_Legality * Vent_size_cm Crosstabulation								
			Vent_size_cm					Total
			no vent (0 cm)	6,05 cm	6,99 cm	7,87 cm	8,64 cm	
Size_Legality	legal_sized	Count	346	368	387	293	214	1608
		Expected Count	454,8	386,4	334,5	255,1	177,1	1608,0
	sublegal_sized	Count	319	197	102	80	45	743
		Expected Count	210,2	178,6	154,5	117,9	81,9	743,0
Total		Count	665	565	489	373	259	2351
		Expected Count	665,0	565,0	489,0	373,0	259,0	2351,0

Chi-Square Tests			
	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	153,364[a]	4	,000
a. 0 cells (0,0%) have expected count less than 5. The minimum expected count is 81,85.			

Symmetric Measures (size effect, strength of association)					
		Value	Asymptotic Standard Error[a]	Approximate T[b]	Approximate Significance
Ordinal by Ordinal	Gamma	-,379	,029	-12,336	,000

Results and evaluation:
χ^2 =153,364, *df*= 4 (2-1)*(5-1), p=0,000<0,05, H_0: rejected. The size legality and vent size are not independent, there is some association. To asses the strength of association, size effect, (categories ordinal, contingency table as 2x5 and H_0: rejected) Goodman & Kruskal's Gamma was used (-0,379). The direction of association negative, strength is moderate (0,379) and significant (p=0,000) When the vent size getting larger legal and sublegal sized fish count decreased. In management point of view, larger vent sizes is benefical (smaller fish can escape) but the catch yield must be considered for legal sized fish, therefore further post hoc tests, twin comparations are needed.

4.2.3. Fisher's Exact Test on fish peduncle spotting (Example 4.2.3.)

In a study, a certain fish species has a morphologic feature "*spots on peduncle*" and it might be related to sexual dimorfism. Total 25 specimens were collected, sex determined, spots in both sides were counted (data are hypotethic). H_0: the spots on peduncle and sex of fish (male and female) are independent H_1:the spots and sex are not independent.

The variables, their carecteristics, frequencies were put a SPSS data file and the FET was applied (because sample is small, some cells expected count less than 5). In SPSS data window DATA> weight cases (frequency) than ANALYZE > DESCRIPTIVE STATISTICS > CROSSTABS in CROSSTABBS window > drag sex to row and spotting to column than click in Statistics window > click Chi-square >CONTINUE > click EXACT, in EXACT window Assimtotic Only is prefered. The size effect (strength of association) and direction measures choosen by nominal or ordinal categories (here categories are nominal, but in this example H0: accepted there is no need further analysis)(IBM SPSS, 2017). Then click CONTINUE> and OK the FET result with other test score and *p* values are shown in Table 4.5. FET can be applied by using =HYPGEOM.DIST(value

in individual cell, total column count, total row count, total sample size, TRUE) in Excel, but it needs excess time and attention.

Table 4.5. In certain fish species sex x peduncle spot cross tabulation, and FET rsults.

Sex * Spot Crosstabulation				
		Spot		
		Spots in left side	Spots in right side	Total
Sex	Male	4	3	7
	Female	8	10	18
Total		12	13	25

Fisher Exact Test FET					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	,326[a]	1	,568		
Continuity Correction[b]	,016	1	,901		
Likelihood Ratio	,326	1	,568		
Fisher's Exact Test				,673	,450
a. 2 cells (50,0%) have expected count less than 5. The minimum expected count is 3,36.					
b. Computed only for a 2χ2 table					

Results and evaluation:
We have good evidence to perform FET that 2 cells (50,0%) have expected count less than 5 (the minimum expected count is 3,36) and sample size small (25). Two-sided exact significance (p)= 0,673 >0,05 we accepted H_0: The spots on peduncle and sex of fish (male and female) are independent (no association between sex and spotting). As seen in the Table 4.5. Pearson χ^2 test (p= 0,568) and Yates' (continuity) correction (p= 0,901), also produce the same results, *p* values definetly higher of 0,05. As H_0: accepted no size effect and direction analysis needed.

4.2.4. Fish consumption preferences in Turkey's costal regions (Example 4.2.4.)

In a study, fish consumption preferences in Turkey's costal regions (Marmara-Black sea, Aegean, Mediterranean) were estimated based on questionaries. Among the items, the response to the question "*have you bought aquaculture products sofar?*" to estimate the regions preference on aquaculture products were tabulated in a 2x3 contingency table (Table 4.6.) and presented in Figure (4.3) (Sagun and Saygı, 2021). After weighting responder number in DATA, the independence test was performed by SPSS as explained before (IBM SPSS v. 26). Adjusted residuals, Phi and Cramer's V (nominal variables) were choosen. The outcomes of the test are seen in Table 4.7.

Table 4.6. Aquaculture product preferences of Turkey's costal regions (Sagun and Saygı, 2021)

Respons	Responders	Marmara-Blacksea	Aegean	Mediterranean
Yes	402	142	151	109
No	248	109	68	71
Total responders	650	251	219	180

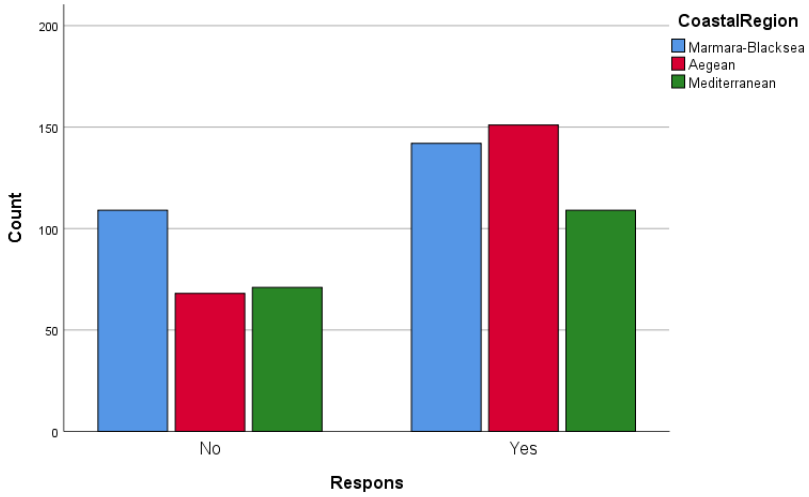


Figure 4.3. Aquaculture product preferences of Turkey's costal regions

Table 4.6. Aquaculture product preferences of Turkey's costal regions cross tabulation, χ^2 and size effect test results

Respons * CoastalRegion Crosstabulation						
			CoastalRegion			
			Marmara-Blacksea	Aegean	Mediterranean	Total
Respons	No	Count	109	68	71	248
		Expected Count	95,8	83,6	68,7	248,0
		% within Respons	44,0%	27,4%	28,6%	100,0%
		Adjusted Residual	2,2	-2,7	,4	
	Yes	Count	142	151	109	402
		Expected Count	155,2	135,4	111,3	402,0
		% within Respons	35,3%	37,6%	27,1%	100,0%
		Adjusted Residual	-2,2	2,7	-,4	
Total		Count	251	219	180	650
		Expected Count	251,0	219,0	180,0	650,0
		% within Respons	38,6%	33,7%	27,7%	100,0%

Chi-Square Tests			
	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	7,767[a]	2	,021
Likelihood Ratio	7,855	2	,020
a. 0 cells (0,0%) have expected count less than 5. The minimum expected count is 68,68.			

Symmetric Measures (size effects, strength of association)			
		Value	Approximate Significance
Nominal by Nominal	Phi	,109	,021
	Cramer's V	,109	,021

Result and interpretation:

χ^2 = 7,767, *df*=2; *p*=0,021 <0,05) H_0: rejected H_1 accepted, aquaculture product preference of coastal regions of Turkey not independent. As Phi=0,109, Cramer's V= 0,109 and *p*=0,021 association is significant but small. If we consider Adjusted residuals (>< 1,96) Yes/no answer relate to Marmara-Blacksea and Aegean coast while Mediteranean not. Appearently Marmara-Black sea and Aegean coasts people prefer aquaculture product a bit more than Mediteraanean.

4.3. Homogeinity

4.3.1. Homogeneity test of bluegill sunfish length frequency (Example 4.3.1.)

The χ^2 homogeinity test is commonly used to test the different of length-frequency distribution. In this example the homogeneity of length frequencies of bluegill sunfish (*Lepomis macrochirus*) that obtained by electrofishing in a sport fisheries pond in years of 1996, 1998 and 2000 (Neumann and Allen 2007). The fish were classified two length group: a) stock to quality size 80-149 mm b) quality size >150 mm (Table 4.7. and Figure 4.4). We want to test the length frequencies homogeneity among the years.

Table 4.7. Size categories of bluegill catch in sampling years (Neumann and Allen 2007)

	Year category		
Size groups/category	1996	1998	2000
Stock to quality size (8—150 mm)	77	124	251
Quality size (>150 mm)	85	44	34

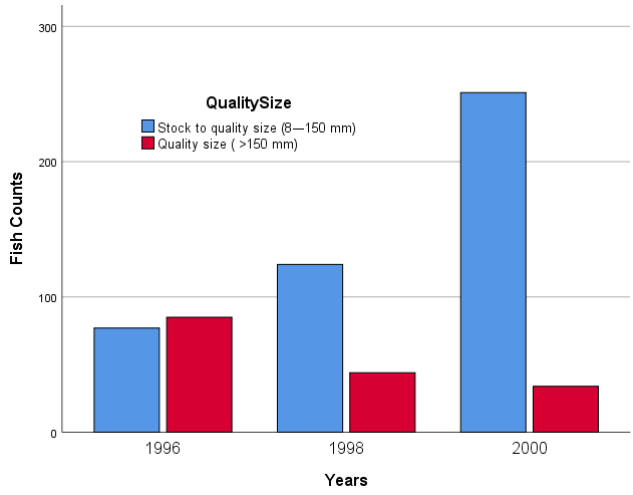


Figure 4.4. Bluegill size categories in the years

H_0: the length frequency distributions of bluegill are homogenous among the years H_1: the length frequency distributions of bluegill are not homogenous among the years; it differs. Alpha (α) was chosen as 0,05 and χ^2 test is performed with SPSS following steps: Variables and their characters were put, then the values weighted (DATA>weight cases, fish count). Next, ANAYZE>DESCRIPTIVE STATISTIC> CROSS TABBS in this stage transfer years to row and fish's size group to column places, choose display clustered bar cards (optional). Then click STATISTIC> In crosstabs: statistics choose Chi Square, Gamma (variables ordinal) than click CONTINUE; click Cell than choose expected counts, percentages of rows or columns, in residual section choose adjusted standardized. CONTINUE > OK. Following outcomes (Table 4.8) were presented.

Table 4.8. Bluegill size categories cross tabulation, χ^2 and size effect test results.

			QualitySize		Total
			Stock to quality size (8—150 mm)	Quality size (>150 mm)	
Years	1996	Count	77	85	162
		Expected Count	119,1	42,9	162,0
		% within Years	47,5%	52,5%	100,0%
		% within QualitySize	17,0%	52,1%	26,3%
	1998	Count	124	44	168
		Expected Count	123,5	44,5	168,0
		% within Years	73,8%	26,2%	100,0%

		% within QualitySize	27,4%	27,0%	27,3%
	2000	Count	251	34	285
		Expected Count	209,5	75,5	285,0
		% within Years	88,1%	11,9%	100,0%
		% within QualitySize	55,5%	20,9%	46,3%
Total		Count	452	163	615
		Expected Count	452,0	163,0	615,0
		% within Years	73,5%	26,5%	100,0%
		% within QualitySize	100,0%	100,0%	100,0%

χ^2 test			
	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	87,154[a]	2	,0001
Likelihood Ratio	85,517	2	,0001
a. 0 cells (0,0%) have expected count less than 5. The minimum expected count is 42,94.			

Symmetric Measures, size effect and direction					
		Value	Asymptotic Standard Error[a]	Approximate T[b]	Approximate Sig.
Ordinal by Ordinal (*)	Gamma (*)	-,615	,051	-9,185	,000

(*) The Gamma statistic (-1 to +1) is a measure of ordinal association between two variables. It measure the strength and direction of association, and it's appropriate in case of contingency table larger than 2x2.

Result and interpretation

χ^2 = 87,154, *df*=2; p=0,0001 <0,05) H_0: rejected H_1 accepted, the size compositions of bluegill catch were not homogenous among years. As size group and years are ordinal, table is (3x2), Gamma value was estimated as -0,615 and *p*=0,000. It means, the years passed the size categories declined (strong negative association), quality sized fish (>150 mm) decrased. In other words, passing the years the counts and percentages of “quality” sized fish getting decrease wheras “stock to quality” fish counts are increase.

4.4. Confounding Factor Analyze

4.4.1. CMH test of new aqua drug experiment (Example 4.4.1.)

In an experimental farm, raised fish were exposed to a new invented aqua-drug to cure a certain parasitic disease in cold A (10-15°C) and warm (B (16-21°C) temperature ranges, not drug exposed fish were used as control. The individulas were sampled randomly to estimate the new drug's effect on diseases (diseased/not diseased). We aim to test dependency between drug and disesase. Besides, it is not clear that the drug's success can be affected water temperature, becase temperature affect both the parasite and fish (it may be a confounding variable). The results were taken for each temperature interval (stratum, *k*) row (drug exposure, r) and column (disease case, c). The 2×2 x *k* contingency tables were prepared and pooled to as one (Table 4.9, Figure 4.5), based on a hypotethic example. The test was performed with SPSS and can be performed also manually by the formulas (sec. 2.4.) using Excel sheets.

Table 4.9. New aqua-drug experiment results contingency table (hypotethic example)

Temperature range	Drug exposure	Diseased	Not diseased	Total
A (10-15ºC)	Exposed	8	24	32
	Not exposed	15	14	29
B((16-21ºC)	Exposed	10	17	27
	Not exposed	22	3	24
Total	Exposed	18	41	59
	Not exposed	37	17	54

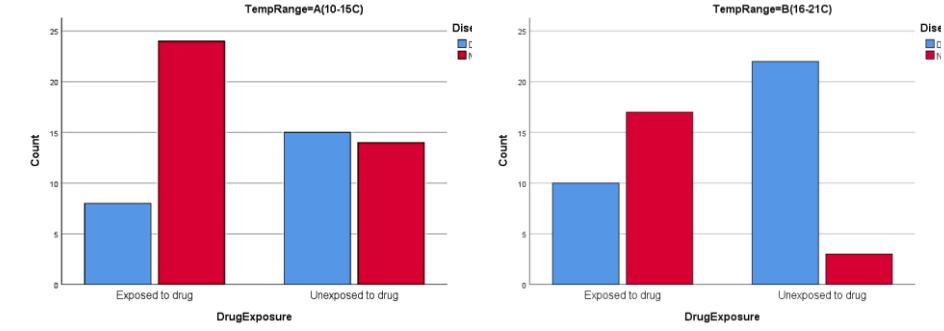


Figure 4.5. The results of new aqua-drug experiment

In SPSS, the varibles are determined, coded, data inserted and weighted to frequencies. Then ANALYZE>DESCRIPTVE STATTISTICS >CROSS TABS> in CROSSTABS dialog window transfer drug exposure to row, disease status to colum and temperature range to layer (confounding), choose cluster bar cart (optional). Then click STATISTICS, in this window, Phi and cramer's V, Cochran and Mantel Heazensel statistic and test common odds ratio = 1 are choosen. Optionally click cell in the dialog box choose expected, row, column percentage click CONTINUE and OK the following outcomes are presented:

Table 4.10. Cross tabulation, χ^2 and CMH test results of new aqua-drug experiment.

DrugExposure * DiseasesCase * TempRange Crosstabulation						
				DiseasesCase		
TempRange				Diseased	Not diseased	Total
A(10-15C)	DrugExposure	Exposed to drug	Count	8	24	32
			Expected Count	12,1	19,9	32,0
			% within DrugExposure	25,0%	75,0%	100,0%
		Unexposed to drug	Count	15	14	29
			Expected Count	10,9	18,1	29,0
			% within DrugExposure	51,7%	48,3%	100,0%
	Total		Count	23	38	61
			Expected Count	23,0	38,0	61,0
			% within DrugExposure	37,7%	62,3%	100,0%
B(16-21C)	DrugExposure	Exposed to drug	Count	10	17	27
			Expected Count	16,6	10,4	27,0
			% within DrugExposure	37,0%	63,0%	100,0%
		Unexposed to drug	Count	22	3	25
			Expected Count	15,4	9,6	25,0
			% within DrugExposure	88,0%	12,0%	100,0%

	Total		Count	32	20	52
			Expected Count	32,0	20,0	52,0
			% within DrugExposure	61,5%	38,5%	100,0%
Total	DrugExposure	Exposed to drug	Count	18	41	59
			Expected Count	28,7	30,3	59,0
			% within DrugExposure	30,5%	69,5%	100,0%
		Unexposed to drug	Count	37	17	54
			Expected Count	26,3	27,7	54,0
			% within DrugExposure	68,5%	31,5%	100,0%
	Total		Count	55	58	113
			Expected Count	55,0	58,0	113,0
			% within DrugExposure	48,7%	51,3%	100,0%

Chi-Square Tests (*)

TempRange		Value	df	Asym. Sig.(2-sided)	Exact Sig. (2-s)	Exact Sig. (1-s)
A(10-15C)	Pearson Chi-Square	4,626[c]	1	,031		
	Continuity Correction[b]	3,558	1	,059		
	Likelihood Ratio	4,680	1	,031		
	Fisher's Exact Test				,038	,029
B(16-21C)	Pearson Chi-Square	14,244[d]	1	,000		
	Continuity Correction[b]	12,172	1	,000		
	Likelihood Ratio	15,352	1	,000		
	Fisher's Exact Test				,000	,000

Total	Pearson Chi-Square	16,305[a]	1	,000		
	Continuity Correction[b]	14,819	1	,000		
	Likelihood Ratio	16,715	1	,000		
	Fisher's Exact Test				,000	,000

a. 0 cells (0,0%) have expected count less than 5. The minimum expected count is 26,28.

b. Computed only for a 2χ2 table

c. 0 cells (0,0%) have expected count less than 5. The minimum expected count is 10,93.

d. 0 cells (0,0%) have expected count less than 5. The minimum expected count is 9,62.

(*) significant association between drug exposure and disease case in A)p=0,033 B) p=0,000 and total p=0,000

Symmetric Measures(size effect)(*)				
TempRange			Value	Approximate Significance
A(10-15C)	Nominal by Nominal	Phi	**-,275**	**,031**
		Cramer's V	**,275**	**,031**
B(16-21C)	Nominal by Nominal	Phi	**-,523**	**,000**
		Cramer's V	**,523**	**,000**
Total	Nominal by Nominal	Phi	**-,380**	**,000**
		Cramer's V	**,380**	**,000**

(*) Phi and Cramér's V are measures of association between drug exposure (nominal) and disease case (nominal) after a χ^2 test family (including CMH) to assess the strength of the relationship. Considering Phi and Cramér's V there are moderate relation between drug exposure and disease (-0,380 and 0,380) in A and total; strong realation in B (-0,523 0,523). It implies warmer temperature increase the relation.

Tests of Homogeneity of the Odds Ratio(*)			
	Chi-Squared	df	Asymptotic Significance (2-sided)
Breslow-Day	**2,226**	1	**,136**
Tarone's	**2,222**	1	**,136**

(*) Breslow-Day and Tarone's tests show odd ratios across strata are homogeneous (i.e., consistent among them, CMH test application is reasonable; the strata results could be pooled). Breslow-Day (χ^2 = 2,226; *df*=1 *p*=0,136>0,05) and Tarone's (χ^2 = 2,222; *df*=1, *p*=0,136> 0,05).

Tests of Conditional Independence (CMH test)			
	Chi-Squared	df	Asymptotic Significance (2-sided)
Cochran's	**17,166**	1	**,000**
Mantel-Haenszel	**15,321**	1	**,000**

Mantel-Haenszel Common Odds Ratio Estimate (OR_{MH})			
Estimate			**,184**
ln(Estimate)			-1,691
Standard Error of ln(Estimate)			,428
Asymptotic Significance (2-sided)			,000
Asymptotic 95% Confidence Interval	Common Odds Ratio	Lower Bound	,080
		Upper Bound	,427

Result and interpretation: To answer the question "*is confounding factor's effect significant?* Several ways can be used: *a) Use of CMH test results*. There is no conditional independence that tested by CMH (χ^2 =15,321 *df*=1, *p*=0,000<0,05), H_0 rejected (H_0: no association between drug exposure and diseasese (outcome) across strata (tepretature range). Drug exposure and parasitic diseases of fish relate to temperature range. The temperature ranges effected of drugxdiseases relation (there is a significant association between temperature and aqua-drug's healing effect, seemingly colder tempretaure range (A 10-15ºC) is more effective to cure parasitic disease than (B 16-20ºC). as the temperature rise aqua-drug's effect is lovered. *b) OR_{MH} value is calculated* as 0,1843 its *p* =0,000 <0,05 the OR_{MH} is statistically significant. It means the temperature range A is 0,184 (%18) fold effective

as the range B. *c) In case of no p value estimation if* OR_{MH} within CI%95 H_0 accepted otherwise rejected.

d) *Comparing crude (unadjusted) odds ratio (OR) with Adjusted OR (*OR_{MH}*)* If there's a substantial difference between the crude OR and OR_{MH}, then the confounder is having a notable effect. SPSS supplies us the first two necessities and we consider these, c and d can be used for manunel computation (crude OR and OR_{MH}).

4.4.2. CMH test of hook types experiment (Example 4.4.2.)

When studying the relationship between fish catches and different types of hooks, another variable may be associated with the catches, so true relationship between catches and different types of hooks is masked. These confounding (masking) variable may be size composition, fishing area, season, temperature, etc. Quaggio et al. (2011) was used CMH to test the efficiency of a particular treatment (e.g. does a circle hook increase the swordfish catch?). In the experiment as a control the J hook also used (contingency table was not presented here). CMH procedure was applied with SPSS to the swordfish catch data obtained from the longline hook selectivity experiments in Brasilian waters of the Southwestern Atlantic from 2004 to 2008. (Quaggio et al. 2011). In result, significant difference was found between two hooks tested (MH $\chi^2_{statistic}$ (10,104) > MH $\chi^2_{critical}$ (3,8) ($p<0,01$). The OR_{MH} for J hook calculated as 1,2 ((833/105055)/(715/106219) that means J hook catching 1,2 fold more swordfish than the circle C hook. The given ratio is crude one (OR), CMH test in SPSS can supply adjusted OR_{MH} as called estimated (Table 4.10. OR_{MH}=0,184)

5. Discussion and Conclusions

χ^2 test family contains Pearson Chi Square, Yates' continuity correction, likelihood ratio (not explained here), Fisher's Exact test and Cochran Mantel Haenzsel tests. The test family are commonly used for Goodnes of fit, independency, homoegeneity, confounding factor seeking tests of categorical variables. All tests are appropriate to use fisheries and aquaculture researches and studies. The type of χ^2test to be used depends on the purpose of the study, sample size, and the number and type of categories.

When we try to compare proportions of a categorical outcome according to different independent groups, we can use Pearson χ^2 and Fisher's exact test. The χ^2test and Fisher's exact test can assess for independence between two variables when the comparing groups are independent and not correlated. The χ^2 test applies an approximation assuming the sample is large, while the Fisher's exact test runs an exact procedure especially for small-size samples.

The Pearson $\chi 2$ test is an approximate method and becomes more accurate as the counts in the cells of the table (RxC) get larger. Therefore, it is important to check that the counts are large enough to result in a trustworthy *p*-value. We can safely use the $\chi 2$ test with critical values from the $\chi 2$ distribution when no more than 20% of the expected counts are less than 5 and all individual expected counts are 1 or greater. In particular, all four expected counts in a 2 x 2 table should be 5 or greater.

The $\chi 2$ test family need some assumptions and with the design of the experiment. χ^2 test base on continuity assumption, if the data are not complied this, Yates' correction should be applied for ensuring contitiuty especially in small sample sizes and small counts. In larger samples, the Yates' correction, Perason χ^2 and FET produce very close *p* values (Table 4.5).

Some problems are identified in GoF test as defining the frequency classes, calculating the $\chi 2$ statistic, and applying the test. Possible solutions for these problems are rewieved intensively by Bolboaca et. al (2011) and others (Mehta and Patel, 2013, McDonald, 2014. Argesti, 2017, Kim 2017). χ^2can be used as GoF test of checking normality (Table 4.1), but presently other normality test methods (Kolgomorov-Simirnov, Shaphiro-Wilk, Anderson Darling) are used widely and many softwares supply them.

The Fisher exact test proved to be the "golden test" in analyzing the independence. But appliying FET by hand calculation is relatively difficult and time consuming, therefore its usage needs statistical softwares. Statistical packages provide the other χ^2 family tests together. The researcher should use the Fisher's exact test of independence in case two categoric (nominal x nominal, ordinal x ordinal, nominal x ordinal or vice versa) variables when the sample size small and some cells count not complied the assumtions (McDonald, 2014).

The Cochran-Mantel-Haenszel procedure should be applied to express confounding effects of other factors such as repetition of trial, seasons, sites, catch-bycatch, environmental variables etc.) CMH test is useful and informative in fisheries, ecology, aquaculture and environmental studies where sampling is made across multiple conditions/habitats/seasons/farms etc.

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Microbial Transglutaminase in Seafood Processing: Sustainable Protein Modification and Valorisation Strategies

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ABSTRACT

Microbial transglutaminase (MTGase) has become a versatile biocatalyst that can significantly change sustainable seafood processing. Its ability to catalyze covalent cross-linking between glutamine and lysine residues in proteins allows for the restructuring of fragmented or underused fish parts into cohesive, high-quality products with better texture and water retention. This chapter explores the biochemical mechanisms, industrial uses, and functional benefits of MTGase in both traditional and new seafood systems, including clean-label, reduced-sodium, and plant-based products. By helping to add value to protein-rich by-products, MTGase promotes circular bioeconomy practices. It enables the creation of edible films, restructured fillets, and bioactive peptides from seafood leftovers. The enzyme's compatibility with alternative proteins like soy and pea makes it relevant for hybrid and vegetarian seafood alternatives. However, there are challenges related to digestibility, allergenicity, and regulatory transparency that are important to consider, especially for sensitive groups. New strategies such as enzyme engineering, fermentation optimization, and integration with non-thermal technologies aim to broaden MTGase's industrial use. Overall, MTGase serves as an important tool for innovation, sustainability, and functional food development in modern seafood processing.

Keywords – Microbial transglutaminase; seafood processing; fish by-products; protein cross-linking; clean-label; sodium reduction; edible films; surimi; functional ingredients; sustainability

INTRODUCTION

The global demand for protein-rich and sustainably produced foods has driven new ideas in food processing technologies. In this context, microbial transglutaminase (MTGase) has become a useful enzyme for improving the quality, structure, and nutritional value of protein-based food products, especially those from seafood and agro-industrial by-products (Kuraishi et al., 2001; Gaspar & de Góes-Favoni, 2015; Yokoyama et al., 2004).

MTGase, mainly derived from Streptomyces mobaraensis, helps form covalent ε-(γ-glutamyl)lysine bonds between protein molecules. This cross-linking improves gel formation, elasticity, and ability to hold water. These features are especially useful in restructuring applications like surimi-based products, fish cakes, and hybrid seafood formulations (Motoki & Seguro, 1998; Andrés-Bello et al., 2011). Additionally, MTGase helps reduce or

eliminate synthetic additives, salt, and phosphates. This supports creating clean-label and low-sodium products (Campagnol et al., 2011; Amirdivani et al., 2018).

The seafood processing sector has a large amount of protein-rich by-products, including fish skin, bones, trimming cuts, and collagen-rich tissues. This sector offers a great chance for MTGase-based strategies to add value. By improving the functional and binding properties of these underused parts, MTGase can help lower waste and support circular bioeconomy models. This is especially important in coastal and aquaculture-heavy regions that aim to increase product value while cutting environmental impact.

Recently, the importance of MTGase has grown in developing plant-based and hybrid protein foods. Its cross-linking abilities allow for major improvements in texture and structure in meat and seafood substitutes, particularly when applied to soy, pea, or algal protein mixtures (Huang et al., 2022; Tsai et al., 2024). However, using it in new food systems also raises ongoing concerns about digestibility, potential allergens, and the immune response to enzyme-modified proteins. These topics continue to inspire research and regulatory discussions (Chanarat et al., 2012; Lerner & Matthias, 2015).

This chapter looks at the biochemical mechanisms, functional benefits, and technological uses of MTGase in seafood processing. It focuses on protein restructuring, by-product valorisation, clean-label reformulation, and integration with sustainable food technologies. It also discusses recent innovations, such as applying MTGase in plant-based systems, fermentation-derived matrices, and non-thermal processing technologies. These innovations are examined in the context of future food system resilience and sustainability.

BIOCHEMICAL PROPERTIES AND MECHANISMS OF ACTION

MTGase is a calcium-independent enzyme that helps form ε-(γ-glutamyl)lysine bonds between proteins. This process improves their structure and function. Unlike tissue transglutaminases from animals, MTGase, which is mainly produced by *Streptomyces mobaraensis*, works well in a wide pH range (5.0 to 8.0) and at moderate temperatures (40 to

50°C). This makes it a great choice for industrial food processing (Motoki & Seguro, 1998; Gaspar & de Góes-Favoni, 2015).

The enzyme has a molecular weight of about 38 kDa and an isoelectric point of around 8.9. It stays stable when refrigerated and can handle moderate heat during processing. This allows it to be used in both cold and heat-processed food systems (Motoki & Seguro, 1998; Yokoyama et al., 2004). These features make MTGase especially useful in making surimi, seafood gels, restructured meat products, and increasingly, in new plant-based food products (Huang et al., 2022).

MTGase catalyses a reaction where the γ-carboxamide group of glutamine residues reacts with the ε-amino group of lysine residues in protein chains (Figure 1). This reaction creates a stable isopeptide bond, called ε-(γ-glutamyl)lysine, between two protein molecules (Motoki & Seguro, 1998). This cross-linking strengthens the molecular network within the protein matrix. As a result, it increases gel strength, elasticity, and water-holding capacity (Tang et al., 2006).

The enzyme specifically targets substrates that are rich in glutamine and lysine residues, such as myosin heavy chain, actin, and various plant proteins, including soy. Importantly, MTGase does not need calcium ions to work. This gives it a significant advantage over animal-derived options in various food systems (Gaspar & de Góes-Favoni, 2015).

Several factors can affect the activity of MTGase, including enzyme concentration, substrate composition, and incubation time. Using too much enzyme or extending reaction times can cause excessive cross-linking, which may lead to protein clumping and lower digestibility. So, careful adjustment is important based on the desired outcome (Tang et al., 2006; Chanarat et al., 2012).

Commercial MTGase is usually made through microbial fermentation with Streptomyces strains. This method allows for high-yield, animal-free production, which supports halal and kosher certification and increases its market acceptance. The enzyme can be applied as dry mixes, dispersions, or injected directly into seafood or meat products. It also works well with plant protein isolates like soy and pea, making it useful in vegetarian and hybrid protein products (Tsai et al., 2024; Zhao et al., 2024).

STRUCTURAL AND FUNCTIONAL IMPROVEMENTS IN SEAFOOD SYSTEMS

MTGase has shown strong effectiveness in changing the structure and flow properties of protein-based foods. Its ability to create bonds allows for the linking of glutamine and lysine residues in nearby proteins. This process strengthens protein networks and improves textural qualities like elasticity, cohesiveness, and water retention (Motoki & Seguro, 1998; Gaspar & de Góes-Favoni, 2015).

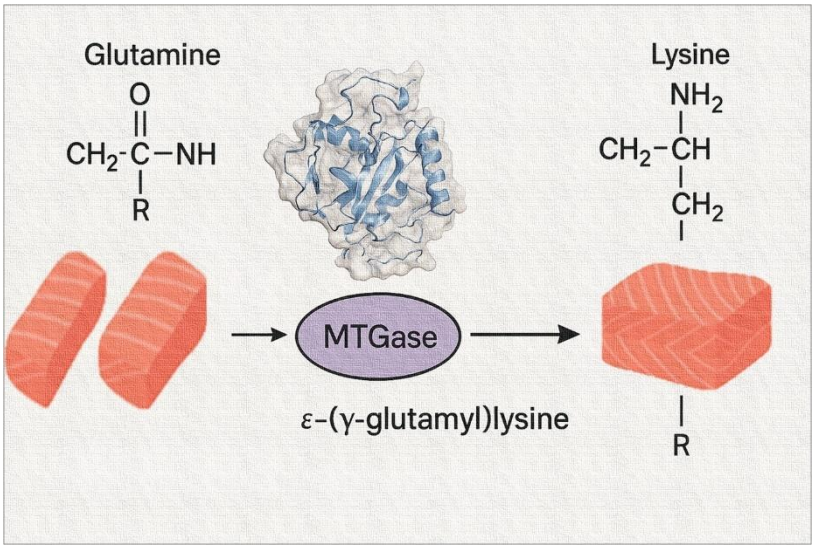

Figure 1. Schematic diagram of MTGase activity on reconstruction of fish fillets.

In seafood processing, MTGase is commonly used in restructured products like fish patties, fish balls, surimi gels, and imitation seafood (Figure 1). It improves the binding ability of fragmented proteins, which helps form a compact, elastic gel. For example, MTGase-treated surimi shows increased breaking force and gel strength from better myosin cross-linking (Seki et al., 1998; Liang et al., 2020). The textural improvement of restructured fish fillets made from fish processing byproducts, such as trims and meat pieces, shows better texture compared to original fillets (Çiçek & Künili, 2025). When mixed with plant protein isolates like soy protein isolate (SPI), MTGase further enhances gelation and texture, making it closer to natural muscle tissue (Kudre et al., 2013; Zhao et al., 2024). MTGase allows gelation at low temperatures, which is essential in seafood

systems that have limited thermal processing options. Cold-set gelation keeps thermolabile nutrients while achieving the desired texture (Andrés-Bello et al., 2011). This feature is particularly helpful when using low-quality raw materials or fragmented fish mince, where the functionality of endogenous myofibrillar proteins may be limited. In meat systems, MTGase is used to bind muscle pieces into cohesive formats like steaks, loins, or sausages. Its ability to cross-link myosin and actomyosin enhances texture and allows for the use of fewer binders like phosphates, promoting clean-label products (Nielsen, 1995; Kuraishi et al., 2001). MTGase is also increasingly useful in plant-based seafood and meat alternatives. When applied to soy and pea protein isolates, the enzyme helps create fibrous, layered structures that visually and texturally mimic shrimp muscle, as shown by scanning electron microscopy (Huang et al., 2022; Tsai et al., 2024). Additionally, MTGase improves the stability of protein-based products during frozen storage (Tokay et al., 2017). Its cross-linking action boosts water retention, reduces syneresis, and prevents structural breakdown during freeze-thaw cycles. Research on surimi and fish mince products indicates that MTGase-treated samples maintain better juiciness and integrity after thawing (Park et al., 1997; Thawornchinsombut & Park, 2006). The dense protein structures formed also reduce drip loss and enhance the thermal stability of emulsions, making MTGase particularly useful in frozen and ready-to-eat seafood products (Altan et al., 2023). However, using too much MTGase can lead to over-cross-linking, which causes protein aggregation, reduces elasticity, and lowers digestibility (Gautam et al., 2025). Over-stabilized protein structures may resist enzymatic digestion, limiting amino acid release during gastrointestinal transit. Therefore, it is important to optimize enzyme concentration, activation time, and substrate composition to meet the product's sensory and nutritional needs (Tang et al., 2006). In conclusion, MTGase is a versatile enzyme that enhances the structure and functionality of protein-rich foods. Its wide range of applications in seafood, meat, plant-based products, and frozen systems reinforces its industrial value and highlights its role in driving innovation in sustainable protein processing.

CLEAN-LABEL AND REDUCED-SODIUM APPLICATIONS

The global demand for clean-label and reduced-sodium food products has pushed manufacturers to reformulate processed foods using natural and functional alternatives. In this context, MTGase has gained attention as an enzyme that improves protein functionality while decreasing the need for synthetic additives like phosphates and extra sodium chloride (NaCl) (Motoki & Seguro, 1998; Gaspar & de Góes-Favoni, 2015). Phosphates are often used in seafood formulations to boost water-binding capacity and protein stability. However, health concerns and shifting consumer preferences have increased interest in phosphate-free options. MTGase provides a natural and safe method by helping form covalent protein links. This process enhances gel strength, elasticity, and water-holding capacity without needing synthetic additives (Nielsen, 1995; Seguro et al., 1995).

Research in surimi and restructured seafood systems shows that MTGase-treated gels have better firmness and lower cooking loss, even without phosphates. This meets clean-label standards while keeping product quality (Liang et al., 2020; Singh et al., 2020). High sodium intake is a significant public health issue, prompting efforts to reformulate seafood products with less NaCl. However, salt is important for dissolving myofibrillar proteins and stabilizing protein gels. MTGase can help make up for the functional losses that come with reducing NaCl by enhancing protein-protein interactions and improving matrix cohesion (El-Bakry et al., 2011; Feng et al., 2018). Studies indicate that MTGase works well under low-salt conditions, especially when combined with salt substitutes like potassium chloride (KCl), calcium ascorbate, and magnesium chloride ($MgCl_2$). These combinations help maintain good textural, gelling, and sensory qualities in reduced-sodium seafood products (Gelabert et al., 2003; Campagnol et al., 2011). For example, KCl has been shown to keep gel strength and water-holding ability in MTGase-treated fish myofibrillar proteins, without affecting consumer acceptance (Feng et al., 2018). Additionally, MTGase helps retain moisture in salt-reduced seafood products, limiting syneresis and cooking loss. These factors are crucial for the quality of frozen and ready-to-eat seafood (Tokay et al., 2021; Altan et al., 2023).

Functional plant protein additives like soy protein isolate (SPI) and Bambara groundnut protein isolate (BGPI) are often used with MTGase to

enhance protein network formation in low-sodium seafood systems. These proteins act as both structural fillers and protease inhibitors, helping to reduce protein breakdown and improve matrix stability (Kudre et al., 2013; Zhao et al., 2024). MTGase has been recognized as Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration and is approved for use in food production across many regions without a specific maximum limit (Gaspar & de Góes-Favoni, 2015). However, concerns have been raised about the potential effects of MTGase's protein cross-linking on digestion and allergenicity. Some studies suggest that MTGase-modified gluten could behave like tissue transglutaminase (tTG), which might provoke immune responses in people with celiac disease (Lerner & Matthias, 2015). While these findings mostly relate to gluten-containing products, ongoing evaluation of MTGase's safety profile is crucial.

VALORISATION OF SEAFOOD BY-PRODUCTS AND CIRCULAR ECONOMY APPLICATIONS

The global seafood industry produces a significant amount of by-products, including fish skin, bones, fins, viscera, and blood. These by-products are often underused, even though they have great nutritional and functional value (Çolakoğlu & Künili, 2016). Using MTGase, fish muscle that is fragmented or low-value can be turned into cohesive and elastic products like fillets, fish balls, sausages, and burger patties. This method decreases the loss of raw materials while making undervalued species and by-products more marketable.

Research has shown that fish pastes treated with MTGase, which come from trimmings or minced residues, maintain excellent texture and sensory qualities that are on par with whole-muscle products (Moreno et al., 2016; Altan et al., 2023). MTGase improves myofibrillar protein binding and reduces syneresis, which enhances water retention and yield. This leads to more stable and profitable product formats (Park et al., 1997). These qualities make it especially beneficial for small-scale or artisanal seafood processors who want to optimize resource use and cut down on waste.

In addition to being produced microbially, transglutaminase can be extracted from fish muscle, liver, and other organs. TGase (pTGase) has also been successfully sourced from various species, including Asian seabass,

with activity levels similar to microbial forms (Binsi & Shamasundar, 2012; Zhang & Simpson, 2020; Gautam et al., 2025). MTGase is important in creating biodegradable films and protein-based coatings from seafood processing by-products, especially gelatin and collagen. These films show better tensile strength, improved barriers against oxygen and moisture, and greater thermal stability. This makes them suitable for food packaging and extending shelf life (Tongnuanchan et al., 2011; Tokay et al., 2017).

MTGase has also been used in enzymatic hydrolysis to produce bioactive peptides from fish by-products like heads, skin, and viscera. These peptides have shown antioxidant, antihypertensive, and immunomodulatory effects (Vasić et al., 2023). Utilizing seafood by-products with MTGase supports several Sustainable Development Goals (SDGs), including SDG 12 (Responsible Consumption and Production), SDG 9 (Industry, Innovation and Infrastructure), and SDG 14 (Life Below Water). By turning protein-rich waste into high-value restructured products, bioactive compounds, and biodegradable films, the seafood industry can lessen its environmental impact, keep waste out of landfills, and create new sources of income (Zhu et al., 1995; Singh & Benjakul, 2018).

LIMITATIONS and CHALLENGES

While MTGase has notable technology and sustainability benefits, its use in food systems has limitations that need careful examination. These include possible effects on protein digestibility, allergenicity, and broader regulatory issues, especially in seafood and other high-protein products. A major technical issue is the risk of excessive cross-linking. Moderate enzymatic activity improves gelation and water retention, but high enzyme levels or extended incubation times can create overly dense protein structures that might resist enzymatic digestion. This can prevent the release of essential amino acids during digestion and lower nutrient availability (Zhu et al., 1995; Tang et al., 2006).

Research on surimi-based gels shows that MTGase-treated samples may produce fewer free amino acids during digestion, which could affect metabolic efficiency and feelings of fullness (Chanarat et al., 2012). Therefore, fine-tuning enzyme dosage, reaction time, and substrate composition is crucial to balance better texture with nutritional quality.

MTGase has also raised worries about its potential to change allergenic protein structures. It may mimic the action of tissue transglutaminase (tTG), linked to coeliac disease. Some studies indicate that gluten modified by MTGase might reveal new epitopes that can trigger immune responses in people who are genetically predisposed (Lerner & Matthias, 2015).

Although most of these findings relate to gluten-containing products, the possibility of unintended immune reactions or increased intestinal permeability in seafood or hybrid protein systems cannot be ruled out completely. In some cases, MTGase could affect tight junctions in the gut, possibly worsening inflammation in individuals with gut disorders like Crohn's disease or type 1 diabetes (Roberts et al., 2013). So far, no clear link has been established between dietary exposure to MTGase and the development of autoimmune conditions. Its Generally Recognised as Safe (GRAS) status and widespread use suggest that, if properly formulated and regulated, MTGase poses little risk to the general population (Gaspar & de Góes-Favoni, 2015).

MTGase activity can vary due to environmental and processing conditions, which might limit its effectiveness in some industrial settings. The enzyme works best at a pH between 5.0 and 8.0, but its activity can be inhibited by metal ions like Cu^{2+}, Zn^{2+}, and Pb^{2+}. These ions can attach to the enzyme’s active cysteine residues, decreasing its effectiveness (Motoki & Seguro, 1998). Therefore, strict quality control of raw materials is vital in seafood processing where metal contamination is possible. Additionally, high-pressure processing (HPP), a non-thermal preservation method becoming more common in seafood processing, can negatively impact MTGase performance. Pressures over 80 MPa can change protein structure, making it harder for the target residues needed for cross-linking to be accessible (Ashie & Lanier, 1999; Herranz et al., 2013). Thus, incorporating MTGase into multi-stage food production requires careful adjustment to maintain both enzyme effectiveness and product quality.

Despite its functional benefits, the use of MTGase in food processing has raised concerns about consumer trust and product labeling. Since MTGase is usually regarded as a processing aid rather than an additive, it might not be listed on ingredient labels, depending on local regulations. This lack of transparency can be troubling for health-conscious or allergen-sensitive shoppers. Recent studies show that consumers are increasingly

aware of and concerned about the use of enzyme processing technologies, especially in clean-label and minimally processed foods (Amirdivani et al., 2018). To address these concerns, food producers might explore voluntary labeling or provide more information to ensure transparency about the origin, function, and safety of MTGase.

FUTURE PERSPECTIVES AND EMERGING APPLICATIONS

MTGase has already changed seafood processing and the development of protein-based foods. As global food systems change due to the need for sustainability, health, and consumer preferences, we need more advancements in how enzymes are applied, delivered, and integrated with new technologies. Future research and industry efforts will likely focus on boosting MTGase's functional uses, increasing production efficiency, and ensuring consumer trust in foods that have been modified with enzymes.

The global protein market is shifting toward plant-based and hybrid options. However, legume- and cereal-based proteins have functional limitations, such as poor cohesiveness and low water retention, which create challenges in formulation. MTGase provides a promising solution by enhancing the fibrous structure, gel strength, and chewiness of proteins from soy, pea, wheat, and other new sources (Huang et al., 2022; Tsai et al., 2024). This enzymatic improvement allows for the creation of seafood alternatives that closely replicate the texture of fish muscle, making them more appealing to consumers. Hybrid formulations that mix marine proteins with plant-based ingredients represent a new way to balance nutritional quality and sustainability.

MTGase helps link different proteins together, strengthening the matrix and enabling the co-structuring of various protein systems in products like restructured seafood, sausages, and coated foods (Zhao et al., 2024). Continued improvements in microbial fermentation, metabolic engineering, and synthetic biology are expected to boost MTGase production by increasing yield, thermostability, and substrate specificity. Using genetically modified *Streptomyces* strains, immobilized enzyme technologies, and continuous bioreactor systems could reduce production costs while enhancing enzyme quality and consistency (Yokoyama et al., 2004).

Recent progress in protein engineering and directed evolution has opened up new paths for creating MTGase variants with better performance under tough processing conditions, like low temperatures, acidic environments, or lower salt levels. Custom-made enzyme variants that can perform site-specific or selective cross-linking may allow for precise structuring in allergen-free or functional food applications (Yokoyama et al., 2004).

Combining MTGase with non-thermal food processing methods—such as high-pressure processing (HPP), pulsed electric fields (PEF), or ultrasound—could lead to combined benefits in gelation, microbial safety, and nutrient retention, especially in minimally processed seafood (Benjakul et al., 2008; Herranz et al., 2013). The long-term success of MTGase in food innovation will rely on technological advancements, regulatory clarity, and consumer perception.

As enzyme applications become more complex, clear labeling, safety communication, and uniform regulations will be crucial. Regulatory frameworks must balance innovation with health protections, particularly regarding allergenicity, protein digestibility, and applications for vulnerable populations. Educating consumers, along with evidence-based labeling that emphasizes MTGase's natural origin, functional benefits, and environmental advantages, will be important for building trust, especially in clean-label, plant-based, and functional food sectors (Amirdivani et al., 2018).

CONCLUSION

MTGase has become an important enzyme in sustainable seafood processing. It catalyzes the bonds between glutamine and lysine residues. This allows fragmented fish proteins to be restructured into cohesive, elastic, and high-quality products. This enzymatic method improves the texture, taste, and functionality of seafood. It also helps make better use of underutilized processing by-products.

Applications of MTGase fit well with the goals of the circular economy and sustainable food production. By improving protein use, cutting down on waste, and enabling clean-label, low-sodium options, MTGase offers a solution that addresses health and environmental issues. Its versatility across traditional, plant-based, and hybrid food systems, along

with its compatibility with new technologies like HPP and edible film production, places MTGase at the forefront of innovation and ecological responsibility. However, adding MTGase to modern food systems requires careful thought about its limitations. Issues like over-crosslinking, reduced protein digestibility, and potential immunogenicity need ongoing scientific examination, especially in new food formats and sensitive populations. Regulatory changes and open communication will be essential for ensuring the safe, accepted, and responsible use of these enzymatic technologies. Looking forward, MTGase is set to take on a bigger role in sustainable protein innovation. Progress in fermentation, enzyme engineering, and targeted delivery systems will enhance its potential. This includes developing cost-effective MTGase variants from seafood by-products. In conclusion, MTGase is a versatile, scientifically backed, and industry-ready tool for the future of seafood processing and protein product development.

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