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Biological studies and Molecular identification of Blowfly, *Calliphora vicina* Rob-Desvoidy (Diptera: Calliphoridae) in the central of Erbil province.

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ABSTRACT

This research aimed to study the life cycle and duration of each biological stage of the blow fly *Calliphora vicina.* Adult specimens were collected from various locations in the Erbil province and reared on decaying chicken meat. Each female fly produces between 440 and 620 eggs during her lifetime, laying them in clusters of up to 180 eggs. The larvae feed for three to four days under warm, optimal conditions, with the puparium forming two to three days later. The entire life cycle of this insect is completed in approximately 15 days. Morphologically, the adult fly is robust, dark black-blue in color, about 5-12 mm in length, while the immature stages (1^{st} , 2^{nd} , and 3^{rd} larval instars) are vermiform, creamy-white, and feature a distinct cephaloskeleton and varying body lengths, while the Larva 1: Vermiform, pale yellow, length 1.7-3.4 mm. the slits of posterior spiracles were conducted to gather slightly appeared as one part. Larva 11 Vermiform, Milky white –pale yellow, length 4.7-7.8 mm. and sometimes absent dorsally, the slits of the posterior spiracles were clearly found and separated in to two lines; . Larva III: The mature larva milky white, reaches a length 14-19 mm. Female: resembles the male but differs in that the eyes are holoptic, separated from one another by broad frons. The slits of the posterior spiracles were distinctly observed and divided into three lines. Another part of the study focused on identifying molecular identification by studying the sequences of the CO1 gene nitrogenous bases, which included 425bp, and comparing them with the information available at the National Center for Biotechnology Information. It was identified as *Calliphora vicina* and registered with strain SM-1 by accession number PP733158.1 in the NCBI database.

***Keywords*:** Calliphora vicina, Biology, Molecular study , identification , Erbil.

INTRODUCTION

There are 1000 recognized species in the family Calliphoridae of the Diptera order, which is distributed all over the world [24]. The family is referred to as bottle flies (green, blue, or brown) or blowflies. A few family members have significant medical, veterinary, and forensic backgrounds [23]. A live animal may become infected with blowflies due to an infection known as myiasis [9] and [19]. Blowfly adults and larvae have the potential to act as vectors and spread harmful germs [28]. They usually are the first to colonize a carcass, often within minutes of exposure [12] and [13]. Numerous species of blowflies are drawn to dead tissues, where they feed, and they may be employed in maggot therapy [20] and [21]. Because they play a significant ecological role in the breakdown of animal remains, blow fly larvae are valued as a useful tool in forensic entomology for calculating post-mortem intervals (PMI) [14]. Blowfly *Calliphora vicina* Rob.-Desvoidy is a common kind of fly that is perfectly adapted for profiting from human activity. It is sometimes referred to as the urban blue bottle blowfly and generally benefits from human populations. The larvae, often known as maggots, consume mostly carrion or food from animals. In forensic investigations, blowflies are extremely helpful since they are willing to settle on corpses [1, 5 and 8]. The species was recorded only during the winter and spring seasons [7] and [1]. It is an EU synanthropic species frequently showing endophilic tendencies and is a facultative myiasis causer [12]. Adults usually feed on decomposing fruits, decaying meat and feces. Larvae are chiefly necrophagous, develop on decomposing meat. *C. vicina* is a significant decomposition agent that accelerates the decay of dead animals. It is a long-range flyer that actively looks for carcasses to reproduce. Because of its ability to detect dead creatures quickly, it is frequently employed in forensic investigations to determine the time of death [3]. In Iraq, Patton in 1920 the first who recoded the species in Iraq, [18] collected the species from different localities of Iraq, and described some species of the family, with key to isolated the genus and specie.

Materials and Methods

The study was based on over twenty specimens of each stage that were collected between January and March 2024 from various locations in the Erbil Governorate, Kurdistan region, Iraq. The adults were collected using an air net, which caught flies on the flowers or that were observed hovering in the air near the flowers, dead matter, and animal excrement.

Collecting and preparing samples

Adults were captured using entomological nets and transferred to the Department of Plant Protection, College of

Agriculture, University of Salahaddin. Adults are drawn to any foul-smelling decaying material, but carrion is by far the most satisfying—especially during the cooler months—and can become a major annoyance. The eggs are laid on the breeding medium, and within a day and accordingly, the larvae hatch. The samples were then identified using the appropriate keys [15 and 26].

Biological study

Each female was collected from animal carrion, rotting meat, and decomposing organic materials then they were put in (20 cm length with 15 cm depth) plastic containers with nylon mesh covering them for the purpose of laying eggs, 10 g of fresh chicken meat was inserted in each container as described by. The larvae in the first through third instars were immediately assembled, at that time the females were raised on decaying meat in cages until they oviposited, reaching the third instar in 3–10 days.

Morphological study

The morphology of the adult and immature was studied using a dissecting microscope, while the minute parts were studied by preparing a microscope slide, by immersing the live larvae in hot water just below boiling point for killing, and by warming the adult drop in a small beaker containing water to boiling temperature for 4-5 minutes to soften its parts and prevent breaking. In order to prepare slides for microscopical analysis, the larvae and adults were dissected, and the necessary components (the head and abdomen of all stages) were placed in a small beaker filled with10%KOH which was placed on fire with shaking for around 4-5 minutes to dissolve the body's lipids and damage muscles. After that, it was immersed in pure water for 2-3 minutes to neutralize the alkaline. After abdomen the mouth parts were divided into separate sections under a microscope and immersed in ethyl alcohol 25%, they were transferred to ethyl alcohol 50%, 75%, and 100%, respectively, for two minutes at each concentration to remove water. After that, they were placed in xylol for two minutes and finally placed in Canada balsam to prepare slides for investigation [6] and [11].

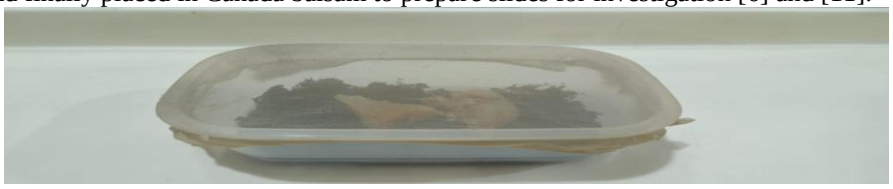


Figure (1): *Calliphora vicina* breeding plastic container

Identification of specimen

Molecular identification

Molecular identification has been utilized to confirm the morphological identification of *Calliphora vicina* which was morphologically determined in general.

Preparation and designing of Primers

Species-specific primer pairs were created for the *Calliphora vicina* blow fly. Rob. -Desvoidy using Primer3Plus website (see to Table 3.2). To prepare a 1000x concentration from the new primers the Sigma molecular biology grade water was added to give a stock of 200pmol/ul. The following vertexing and spinning the tubes were kept on ice for 30 minutes to generate a 10x primer stock; 1 µl of the stock was combined with 9 µl SDW for the Forward and Reverse primers, which were placed in a -20ºC freezer till used for DNA extraction. A stock solution of 200 pmol/µl was created by adding water of Sigma molecular biology grade in order to generate a 1000x concentration from the new primers. To make a 10x primer stock, the tubes were centrifuged, vertexed, and then cooled on ice for half an hour. For the Forward and Reverse primers, one microliter of this stock was combined with nine microliters of sterile distilled water. After that, they were kept at -20°C till DNA extraction was done. Partial cytochrome oxidase I Sequenced gene. The ABI 3130X genetic analyzer (Applied Biosystem) was used to perform independent DNA sequencing with only the forward primer C1-J-1718. PCR products from the sample were used as a source of DNA template for sequenced specific PCR amplification. Larval specimens of *Calliphora vicina* Rob. -Desvoidyv were examined for polymorphisms in their mitochondrial Cytochrome oxidase one (CO1) sequence using a single forward primer(5'GGAGGATTTGGAAATTGATTAGTTCC-3'). The analysis was conducted using an ABI 3130X genetic analyzer from Applied Biosystems. PCR products from 20 samples, generated using the ABI Prism Terminator Sequencing Kit (Applied Biosystems), were employed as DNA templates for sequence-specific PCR amplification at Macro Gene Molecular Company in Korea.DNA Extraction for *Calliphora vicina*. Larvae-containing samples were gathered from a rearing cage within the higher education laboratory at the College of Agricultural Engineering Science in March 2024. These larvae were observed and subsequently utilized in the experiment. All samples were placed in a nylon bag and then collectively stored in liquid nitrogen. Following their immersion in liquid nitrogen, the individuals were crushed into a fine powder and the complete larvae's genomic DNA was extracted using the USA-made ZYMO Quick-DNA Tissue/Insect Micro Prep Kit and Beta Bayern Tissue DNA Preparation Kit (No. D6015). After that, the extracted DNA was kept at -20°C in a freezer until it was needed again. The concentration of extracted DNA from the sample was estimated using a) Nanodrop, 1000 UK) spectrometer.

PCR Amplification of Cytochrome Oxidase subunit 1 (CO1)

Using a Bioresearch PTC-200 Gradient thermocycler, PCR amplification for the CO1 partial gene was carried out in 50 µl of reaction mixture comprising 2x Taq DNA Polymerase Master Mix (AMPLIQON A/S Stenhuggervej 22), as shown in Table1.The primers of specific gene in mitochondria which is known as mt-CO1 gene, was chosen for PCR amplification, [4] using the following pairs of primer that is shown in(Table 2)

Table 1: Reagents for Cytochrome oxidase 1 PCR amplification

No.	PCR components	Concentration	Volume (µl)
1	Master Mix (AMPLIQON A/SStenhuggerve22)	2x	25
2	Forward Primer	20 Pmol	3
3	Reverse Primer	20 Pmol	3
4	DNase free Water	-	15
5	Template DNA	50ng/µl	4
Total			50

Table 2: Steps of PCR amplification

Steps	Temperature ^{O}C	Time	Cycles
Initial denaturation	95 ^{O}C	5 min	1
Denaturation	95 ^{O}C	40 seconds	35
Annealing	60 ^{O}C	40 seconds	
Extension	72 ^{O}C	1 min	
Final extension	72 ^{O}C	10 min	1

Table 3: PCR primer CO1 gene used for molecular study of Calliphora vicina Rob. -Desvoidy

Gene	Nucleotide Sequences	Product size PS (bp)	TM
Cytochrome Oxidase c subunit I (CO1)	forward primer C1-J-1718 5'-GGAGGATTTGGAAATTGATTAGTTCC-3' Reverse primer C1-N-2172(HCO2198) 3'- TAAACTTCAGGGTGACCAAAAAATCA -5'	425bp	60o C

Agarose Gel Electrophoresis and Visualization of DNA fragments

After 30 minutes in the electric field of electrophoresis, an intercalating dye of Ethidium bromide is added to 1.5% melted agarose gel in 1X TAE buffer, and the location of bands is identified by analyzing the gel under a UV transilluminator.**Sequence Alignment and Phylogenetic Analysis**

The PCR products were visualized by exposure to ultraviolet light. Then, the gene products were sent to Korean Macrogen Co. for sequencing. After that the sequencing result was registered and the chromatograms of these incomplete gene sequences were accurately edited, and base calls were confirmed using the Finch TV software program. CO1 partial gene, and sequence alignment was done to compare and align the sequences obtained in this study with those from other biological samples available globally. compared with NCBI-BLAST to identify, the phylogenetic tree was drawn up using MEGA11 v.11.0.13 software's with the number of strains registered in NCBI.All PCR results containing partial CO1 genes were sequenced using the ABI Prism Terminator Sequencing Kit from Applied Biosystems at Macrogene Molecular Company in Korea. This alignment was completed with Bio Edit and MEGA v.11.0.13 software. All sequences generated during this study were deposited in the NCBI-GenBank.**RESULTS AND DISCUSSION**

Biological study

Calliphora produced between 440 and 620 eggs in her lifetime, which are placed in groups of up to 180 eggs at a time. The larvae feed for three to four days in warm, otherwise ideal conditions, and the puparium forms two to three days later. The larvae may feed for up to nine days in colder areas. The pupal stage lasts for at least a week, although in poor circumstances, it can last much longer. The temperate zone's winter is most likely over when the larvae are in the so-called "prepupal stage," which is when they are no longer feeding and have left the breeding medium. The entire life cycle, from egg to egg, takes at least 15 days in general. about 120 hours (5 days) while under 26^{o}C and relative humidity13% they were leave 216 hours about (9days). **Duration of life cycle of *Calliphora vicina* Rob. -Desvoidy**

Egg: The eggs hatching after 25-31 hours under20 ^{o}C humidity %35 and 20-24 hours under 26 ^{o}C and %13 humidity and turned to **1st larval instar**

2nd larval instar: in 20 ^{o}C and humidity %35 after 35 hours the 1st larval instar changed to 2nd larval instar while in 26 ^{o}C and relative humidity %13 the larva was presented after 30 hours

3rd larval instar: the 3rd larval instar presented after 52 hours under 20 ^{o}C and relative humidity %35 while in 26 ^{o}C and relative humidity %35 they were found after 45 hours.

Pupal stage: period pupal stage of *Calliphora vicina* under 20 ^{o}C relative humidity 35% and 26^{o}C relative humidity 13% was

10 and 14 days respectively.

Adult: the adults were allowed to live under 20ºC and relative humidity 35% . Similarly, the eggs hatch after 20-28 hours and turn into the first larval instar, followed by the second instar after 18-34 hours and the third instar after 16-18 hours. The third instar remains for a few days and changes into the pupa, and after two weeks, the adult emerges, according to [16].

Morphological studies of biological stages

Egg: A female lays 200 -220 bright creamy-white eggs, although larger eggs appear to be yellowish (Fig. 1.a). On fresh carrion, the eggs are banana-shaped, white, about 1.3- -1.6 mm long, with a median stripe 1.2-1.4 mm, and the micropyle is very small.

Larva 1: (Fig 1.b) Vermiform, pale yellow, length 1.7-3.4 mm. Head with 4-5 spinose bands ,2^{nd} -7^{th} segments with complete anterior bands; 6^{th}-11^{th} segments with posterior spinose bands, meanwhile slits of posterior spiracles were conducted to gather slightly appeared as one part , Cephaloskeleton slender and weak.

Larva 11: (Fig.2c). Vermiform, Milky white –pale yellow, length 4.7-7.8 mm. The band s wider and composed of larger spines than in the previous instar. 2^{nd}-9th segments with complete anterior spinose band s, while those on the 8^{th}-9th segments weak and sometimes absent dorsally, the slits of the posterior spiracles were clearly found and separated in to two lines ; Cephaloskeleton was determined clearly and well developed.

Larva III :(Fig2.d). The mature larva milky white, reaches a length 14-19 mm. 2^{nd}-9^{th} segments with complete spinose band s, anterior band s not complete dorsally on tenth to twelfth segments; 6^{th}-11^{th} segments posteriorly with spinose bands which are complete on the 9^{th}-11^{th}. The slits of the posterior spiracles were distinctly observed and divided into three lines. Cephaloskeleton very well developed; basal part of mouth hook quadrate, apical part hook like, Accessory oral sclerite oval shaped.

Puparium (Fig.2e): Oval shaped, brown, length 7.1-9.6 mm; each segment with many transverses striates between the spines, each 2^{nd}–3^{rd} segment with 20-25 rows of striates; anther external characters with of the mature larva.

Morphological identification for adult

The adult Calliphora is divided into three equal parts the head, thorax, and the abdomen. *C. vicina* species are metallic blue-black blowflies of about 10-12 mm in length. These flies live in urban areas and are the first to reach corpses. The adult fly has a yellow-brown body and is about 6–12 mm long. Full-grown larvae are 13–15 mm long. The main identification features used in identifying C. vicina are illustrated in [1] Calliphorinae are characterized by the absence of a row of setae on the dorsal surface of the stem vein, the bristly lower calypter and proepisterna depression, and the supra region is bare or with only a few scattered bristles.

Adult: Body: Robust, dark blue, measuring 5-12 mm in length

Male: black, Phallus (Fig.3B) dark brown-black, basiphallus rectangular shaped
epiphallus knife shaped, paraphallus xyphoid, hypophallus dagger.

The female: resembles that of male but differs by the eyes are an holoptic separated from one another by a broad frons. 5^{th} abdominal sterinte oval shaped (Fig.3B)

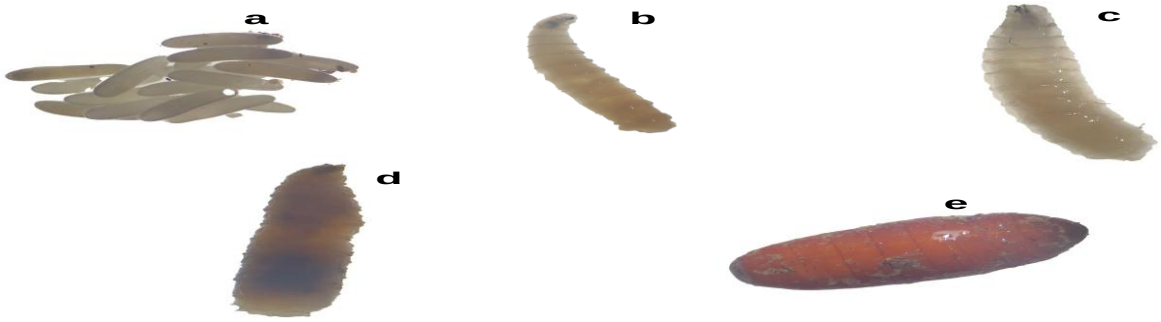


Figure (2): The life cycle of *Calliphora vicina*: a: Egg 12x , b: 1^{st} larval instar 11x , c: 2^{nd} larval instar 6x, d: 3^{rd} larval instar 2x e: Pupa 5x

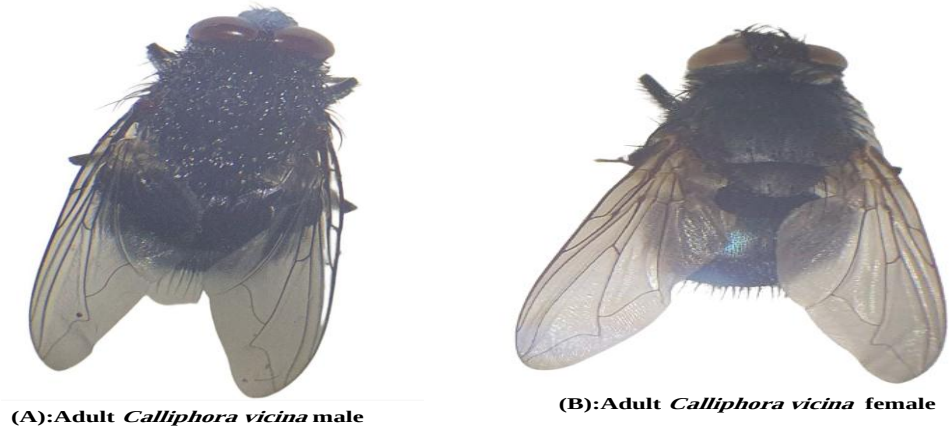


Figure (3): Adult Calliphora vicina: A: male 4x and B: Female: 6x

Molecular Identification

PCR amplification of partial CO1 gene

Species specific primers of Mitochondrion gene-were designed using the sequences of cytochrome c oxidase subunit I Synthesized by South Korean Micro-gene Company the primers could produce a band of 425bp. The PCR product was electrophoresed and visualized by 1.5% Agarose gel figure (4).

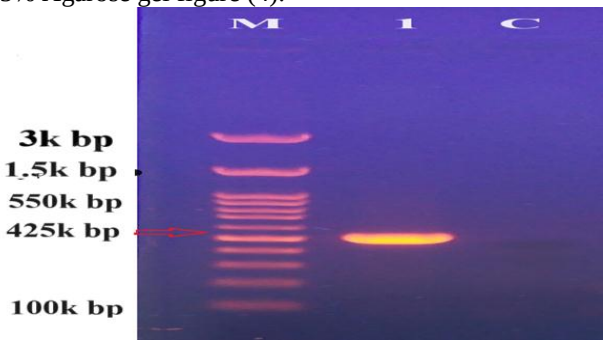


Figure (4): Partial cytochrome Oxidase gene from insect amplified by PCR. M; shows: ladder 100–3000 bp, lane 1: 425 bp of insect PCR products, and C is the negative

Table (4): Percentage Distribution of Samples of Insect Species in to Calliphora vicina According to Blast of GenBank NCBI of Partial CO1 Gene

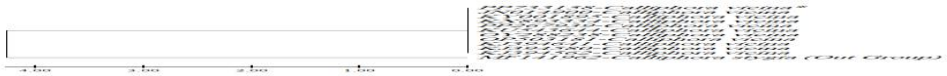
Insect Identified	Accession Numbers	Query Cover %	Identic Number %	Accession Number of BLAST Identification
Calliphora vicina	PP733158	100	100	JN014900
		100	100	KY001895
		100	100	KX893334
		100	100	PP267939
		100	100	KU543644
		100	100	OY288238
		100	100	OP503181
		100	100	KJ394641
		100	100	KJ394580
		100	100	KJ394562

Molecular Identification of Genus and Species of Insect

The BLAST tool from the GenBank (http://blast.ncbi.nlm.nih.gov/) is used to generate the CO1 sequence of insect species from samples with a size of 425 base pairs. was utilized to compare our amplified sequences to previously stored species sequences. BLAST findings showed that the query sequence with the greatest identity was found in the NCBI gene repository for insect identification. These aliments reflect the submission of our query sequences into NCBI GenBank, and the given accession numbers comprise table (4).

Phylogenetic inferences

Phylogenetic study based on CO1 nucleotide sequence indicated grouping of investigated species. *Calliphora vicina*. Rob. -Desvoid of expected lines. According to sequencing divergence similarity data and the ph



ylogeny produced [27], species belonging to respective genera were near to each Figure (5).

Figure (5) shows the phylogenic tree of Calliphora vicina. Rob.-Desvoidy sample from Iraqi Kurdistan region (*). The phylogenic tree was created using MEGA11 software's Maximum Likelihood approach based on the Tamura-Nei model, as well as bootstrap analysis with 100 resampling. Partial DNA sequences of the concatenated partial CO1I mitochondrial gene were used as input data.

Molecular findings from this study revealed that no genetic variation between *C. vicina* species collected from different locations in Erbil. The cytochrome oxidase subunit I (CO1) gene was successfully sequenced from the species with a total product size of 425 bp and analyzed for the purpose of identification Also, particularly given the fact that no gene sequencing has been conducted on this economically important species in Erbil and Kurdistan region. The development of PCR primers capable of amplifying the CO1 barcode region from a diverse species of Calliphora has ensured the widespread use of this region for species discrimination [29]. The species-specific primer with complete query cover has been designed, and the product size was 425 bp for the purpose of species identification, and it was confirmed that the samples belonged to *C. vicina.*

As a result, our findings should facilitate future comparative studies with other Calliphora species from other geographical locations, as they will be stored in GenBank and the Barcode of Life Database as mitochondrial CO1 sequences. On the other hand, the phylogenetic tree of *Calliphora vicina* reveals a distinct grouping of genetic sequences from the NCBI database, except sequence MF141962, which was used as an outgroup. This outgroup status is attributed to the geographical isolation and excessive use of chemicals in the region where this particular sequence was obtained. The geographical isolation likely led to a degree of genetic divergence from other populations, while the high exposure to chemicals may have induced additional genetic variations. As a result, MF141962's placement as an outgroup helps in understanding the evolutionary impact of these environmental factors on the genetic diversity within *Calliphora vicina* and this is with agreement with [17] and [10].

Conclusion

It can be concluded that the blow fly *Calliphora vicina* Rob. -Desvoidy life cycle and how long each biological stage lasted. For this reason, adult specimens were gathered and raised on rotting chicken meat in various provinces of Erbil. As a result, the fly laid between 440 and 620 eggs during her lifetime, the outer appearance of adult is robust, dark black-blue in color, and length approximately 5 to 12 mm. In contrast, the first, second, and third larval instars of this insect are typically vermiform in shape, creamy-white in color, with a distinct Cephaloskeleton and varying body lengths. The other part of the study focused on the molecular techniques for insect identification using PCR, species-

specific primers were created and manufactured. We used the CO1 sequence of samples with a size of 425 bp, which is fed by the BLAST program from Gen bank (http://blast.ncbi.nlm.nih.gov/), to compare our amplified sequences with other stored species sequences. The submitted accession number for this program is PP733158. The NCBI gene bank identity of insects contained the highest identity query sequence, according to the BLAST results. The NCBI gene bank identity of insects contained the highest identity query sequence, according to the BLAST results.

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الدراسة البيولوجية والتشخيص الجزيئي لذبابة البطل الازرق *Calliphora vicina* Rob-Desvoidy (Diptera: Calliphoridae) في محافظة اربيل.

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الخلاصة

يهدف هذا البحث إلى دراسة دورة حياة ومدة كل مرحلة بيولوجية لذبابة ***Calliphora vicina Rob.-Desvoidy*** ولهذا السبب تم جمع عينات بالغة في مواقع مختلفة من محافظة أربيل وتربيتها على لحم دجاج فاسد، وبالتالي أنتجت الذبابة ما بين 440 إلى 620 بيضة. خلال حياتها، والتي يتم وضعها في مجموعات تصل إلى 180 بيضة في المرة الواحدة. تتغذى اليرقات لمدة ثلاثة إلى أربعة أيام في ظروف دافئة أو مثالية، وتتشكل العدراء بعد يومين إلى ثلاثة أيام وتكتمل مدة دورة حياة هذه الحشرة في 15 يومًا بشكل عام. من الناحية الشكلية، تكون لون الحشرة البالغةاسود الى ازرق ويبلغ طولها حوالي 5-12 ملم) في حين أن المراحل غير الناضجة لهذه الحشرة (الأطوار اليرقية الأولى والثانية والثالثة كانت بشكل عام دودية الشكل ولونها أبيض كريمي مع هيكل رأسي مميز و وركز الجزء الآخر من الدراسة على الطرق الجزيئية للتعرف على الحشرة باستخدام طريقة ***PCR***، ولهذا السبب تم إعداد وتصميم بادئات محددة للأنواع. وبالتالي، تم تعزيز تسلسل ***CO1*** للعينات بحجم 425 زوجًا وتم استخدام برنامج جين بانك من ***(//blast.ncbi.nlm.nih.gov)*** برقم الانضمام المقدم ***PP733158*** لمقارنة تسلسلاتنا المضخمة مع تسلسلات الأنواع المخزنة الأخرى. أشارت النتائج التي تم الحصول عليها من بلاست إلى أن أعلى تسلسل استعلام عن الهوية تم تسجيله في هوية بنك الجينات ***NCBI*** للحشرات.

الكلمات المفتاحية : ***Calliphora vicina Rob.-Desvoidy,*** الحياتية ، التشخيص الجزيئي.