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ARTICLE

Surveillance of *Vibrio spp* in *Penaeus monodon* Collected from Shrimp Pond of Satkhira, Bangladesh

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ABSTRACT

Vibrio is the most common genera associated with crustaceans and often causing significant economic losses. Many *Vibrio* species are pathogenic to human and have been implicated in food borne diseases. The present study was conducted to identify *Vibrio* spp. from the tiger shrimp (*Penaeus monodon*) of shrimp pond at Satkhira, Bangladesh. A total number of 33 *Vibrio* species isolates were identified from 20 shrimp samples through a series of morphological, physiological and biochemical tests. The work reports the prevalence of *Vibrio* spp. in the pond environments and the existence of three *Vibrio* species such as V. *alginolyticus*, *V. parahaemolyticus* and *V. harveyi* were identified. In the study of antibiogram, all isolates were shown 100% sensitive to streptomycin, ciprofloxacin and chloramphenicol. Maximum 41% isolates were shown resistant to co-trimethaxozole whereas 30% and 24% resistant to azithromycin and novobiocin respectively.

1. Introduction

Mainly tiger shrimp cultivation is almost exclusively concentrated in three districts of Bangladesh namely, Satkhira, Khulna, Bagerhat (along with Rampal) [1]. Over 70% of the total numbers of farms are located in the greater Khulna (Satkhira, Khulna and Bagerhat), which accounts for 74% of the land area under shrimp cultivation and 77% of total output. The remainder of the farm area under cultivation and their output are almost entirely accounted for by Cox's Bazar. In the major shrimp producing areas, shrimp yields range from between 150 kilogram per hectare in Cox's Bazar and just over 200 kilogram per hectare in Satkhira.

The tiger shrimp farming activities contribute significant role in the national economy of the country. Shrimp is second most important exportable commodities in Bangladesh having a high demand and price in the international market [2- 4]. Besides, its production through aquaculture and trade offers unique opportunity in providing employment and poverty alleviation. The tiger shrimp, *Penaeus monodon* an agricultural product is recognized as a considerable one for its remarkable contribution in soaring the foreign exchange earnings. Fisheries sector contributes 4.57% to the Gross Domestic Product (GDP) and shrimp alone contributes about 0.07% of total export earnings [5].

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Due to the increase in virulence of pathogens, especially bacterial and viral, a crisis in the shrimp industry was affected over the last few years. Low survival rates in hatchery or grow out conditions are often associated with bacterial diseases caused by *Vibrio spp*. Rather the treating the underlying cause, the diseases are treated in this cases. With the use of antimicrobial chemicals, especially antibiotics and chlorine along with the interactions of microbes, animals and their environment under the stress of commercial production of *Penaeus monodon*, have led to the emergence of more virulent pathogens [6]. For the identification of the presence of the toxic chemical, 'nitrofuran and its metabolites' that was used as broad spectrum antibiotic, several shipments of exported frozen shrimp from Bangladesh were rejected by EU, which caused a loss of about US$ 10 million in 2008 in Bangladesh [7].

The present study was conducted on the identification of *Vibrio spp*. in *Penaeus monodon* as it is the main target species for culture and high production in Bangladesh which is one of the important species for animal protein as well as great source for foreign earnings. However, the main barrier of the production of *Penaeus monodon* is pathogenic which is effect caused by the viruses and bacteria. *Vibrio* is one of the important pathogen that reducing production of this tiger shrimp. Very few researches have been done in *Penaeus monodon* with *Vibrio* in Bangladesh whereas no record was found shrimp pond of Satkhira. Therefore, this research is interested to study the observation of *Vibrio* status in *Penaeus monodon* of shrimp pond in Satkhira, Bangladesh.

2. Materials and Methods

2.1 Sample Collection

All of the experiments were carried out in the Zoology, Fisheries and Marine Biotechnology Research Unit in the Department of Genetic Engineering and Biotechnology at Shahjalal University of Science and Technology, Sylhet, Bangladesh. Samples were collected from twenty shrimps, which were selected randomly from 05 shrimp ponds in Katakhali village of Satkhira, Bangladesh. The samples of *Penaeus monodon* (5-9 cm long) were caught by harvesting with net and then transferred into the sterile polyethylene bag. All the samples were carried to the lab as soon as possible with an ice box.

2.2 Preparation of the Sample

All the samples were prepared and kept in different trays swabbed in 70% alcohol. For having normal temperature they were kept in the laminar airflow for 30 min. Bacterial swab from different parts of the inner and outer body were cultured into the *Vibrio* specific TCBS agar.

2.3 Isolation of Bacteria

Isolates with green and yellow colonies from each plate were isolated and sub-cultured into nutrient agar medium (supplemented with 2% NaCl). Bacterial specimen was collected only from single colony of each. All the sub-cultured bacteria were preserved for further biochemical tests and investigation.

2.4 Morphological Characterization

The studied bacterial isolates were provisionally identified on the basis of the results of morphological test such as Gram staining. Gram staining was performed using compound microscope according to the procedure described by Petersen *et al.,* 2016 [8]. The gram positive and gram negative bacteria were identified based on violet and pink color respectively.

2.5 Biochemical Characterization

Various biochemical tests i. e. Catalase test, Oxidase test, Oxidation fermentation (O-F) test, Motility test, Salt tolerance test, Indole test, Methyl-Red (MR) test, Voges-Proskauer (VP) test, Citrate test, Urease test, Gelatin liquefaction test, Dextrose utilization test, Hydrogen sulfide (H_2S) production test and Lysine decarboxylase test were performed to identify bacterial isolates.

2.6 Salt Tolerance Test

With varying amounts of NaCl (0%, 4%, 6%, 8%, and 10%), salt tolerance test was done on nutrient agar media supplemented. To study the salt tolerance range of the isolated species and the optimum concentration, the test was performed.

2.7 Determination of Antimicrobial Sensitivity Patterns

Susceptibility of bacterial isolates to different antibacterial agents was determined in vitro by disc diffusion method as described by Rahman and Hossain (2010) with slight modification. The procedure involved measuring the diameter of the zone of inhibition that results from diffusion of the agent into the medium surrounding the disc. Commercially available eight antimicrobial discs such as streptomycin, erythromycin gentamycin, chloramphenicol, ciprofloxacin, co-trimethxazole, azithromycin, novobiocin were used for the test.

3. Resuls

3.1 Isolation of *Vibrio spp*.

Twenty shrimp samples were screened for observing the

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presence of *Vibrio spp*. All the examined shrimps were found positive with *Vibrio spp*. A total of 33 *Vibrio* spp. isolates were collected from the experimental shrimps. The incidence of *Vibrio spp*. varied depending on the samples examined. The isolates were numbering as H1-H13, H15-H23, H25-H26 and H29-H37.

3.2 Morphological Characteristics of *Vibrio spp*.

Morphological studies were carried out by microscopic observations and growth characteristics in petri dishes. Fifty three yellow or green colored pin point colonies bearing colony characteristics of *Vibrio spp*. were preliminary selected from TCBS agar plates. The isolated colonies were small to medium in size and rounded. Thirty three isolates out of those fifty three were gram negative and short rod shaped. They were able to grow at 25^0C and 37^0C but failed to grow at 4^0C.

3.3 Biochemical Characteristics of the Isolates

A series of biochemical tests were performed to identify the *Vibrio* spp. isolates up to species level (Table 1). All the isolates were positive for oxidase, catalase and O-F test. In indole test H3, H6, H10, H17, H30 and H35 showed negative result while all other isolates showed positive result. The negative results were found for all isolates in methyl-red test except the isolates H6, H10, H15, H20, H30, and H36. Again isolate no. H4, H7, H8, H13, H17, H21, H22, H31 and H33 showed positive result for Voges-Prausker test, whereas others showed negative result. In case of citrate utilization test all isolates were negative except isolates H8, H10, H15, H16, H17, H19, H25, H31 and H35. In urease test only isolates H6, H10, H16,

Table 1. Biochemical characterization of *Vibrio* spp. Isolates

Isola-tes	Tests performed												
	C	O	M	O-F	VP	MR	I	Citrate utili-zation test	Urease test	Gelatin lique-faction test	Dextrose utili-zation test	H2S test	Lysine decarbo-xy-lation test
H1	+	+	+	+	-	+	+	-	-	+	-	-	-
H2	+	+	+	+	-	+	+	-	-	+	-	-	-
H3	+	+	+	+	-	+	-	-	-	+	-	-	-
H4	+	+	+	+	+	+	+	-	-	+	-	-	-
H5	+	+	+	+	-	+	+	-	-	+	-	+	-
H6	+	+	+	+	-	-	-	-	+	-	-	-	-
H7	+	+	+	+	+	+	+	-	-	+	-	-	-
H8	+	+	+	+	+	+	+	+	-	+	+	+	-
H9	+	+	+	+	-	+	+	-	-	+	-	-	-
H10	+	+	+	+	-	-	-	+	+	-	+	-	-
H11	+	+	+	+	-	+	+	-	-	+	-	-	-
H12	+	+	+	+	-	+	+	-	-	+	-	+	-
H13	+	+	+	+	+	+	+	-	-	+	-	-	-
H15	+	+	+	+	-	-	+	+	-	+	-	+	-
H16	+	+	+	+	-	+	+	+	+	-	+	-	-
H17	+	+	+	+	+	+	-	+	+	+	+	-	-
H18	+	+	+	+	-	+	+	-	-	+	-	+	-
H19	+	+	+	+	-	+	+	+	-	+	-	-	-
H20	+	+	+	+	-	-	+	-	+	+	-	-	-
H21	+	+	+	+	+	+	+	-	-	+	-	-	-
H22	+	+	+	+	+	+	+	-	-	+	-	-	-
H23	+	+	+	+	-	+	+	-	-	-	-	-	-
H25	+	+	+	+	-	+	+	+	-	+	-	+	-
H26	+	+	+	+	-	+	+	-	-	-	-	+	-
H29	+	+	+	+	-	+	+	-	-	+	-	-	-
H30	+	+	+	+	-	-	-	-	+	-	+	-	-
H31	+	+	+	+	+	+	+	+	-	+	-	+	-
H32	+	+	+	+	-	+	+	-	-	-	-	-	-
H33	+	+	+	+	+	+	+	-	-	+	-	-	-
H34	+	+	+	+	-	+	+	-	-	+	+	-	-
H35	+	+	+	+	-	+	-	+	+	+	-	-	-
H36	+	+	+	+	-	-	+	-	-	+	-	-	-
H37	+	+	+	+	-	+	+	-	-	+	-	-	-

Notes: C= Catalase test, O= Oxidase test, M= Motility, O-F= Oxidation fermentation test, VP= Voge's-Proskauer, MR= Methyl Red test, I= Indole Test, Hydrogen sulfide (H_2S) production test.

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H17, H20, H30 and H35 were found positive. In gelatinase test maximum isolates were found positive, whereas the isolates H6, H10, H16, H23, H26, H30 and H32 were showed negative. Only six isolates (H8, H10, H16, H17, H30 and H34) were showed positive in dextrose utilization test, otherwise rests of the isolates were found negative. There are eight isolates (H5, H8, H12, H15, H18, H25, H26 and H31) were given positive result in H_2S test. All isolates were found negative result in lysine decarboxylase test.

3.4 Salt Tolerance Test of *Vibrio* spp.

All the isolates were grown in nutrient agar media containing 4%, 6%, 8% and 10% NaCl. No isolates were grown in 0% NaCl (Table 2).

3.5 Observed Species Diversity

Three *Vibrio* species such as *Vibrio Alginolyticus*, *Vibrio parahaemolyticus* and *Vibrio harveyi* were identified. It was found that isolates H4, H7, H13, H21, H22 and H33 were motile, Gram negative, positive in catalase and oxidase test, positive in MR and indole test (Table 01). Besides these isolates were positive in VP, gelatinase and oxidase-fermentation test but were negative in urease test and were identified as *Vibrio. Alginolyticus* (Table 01). This study was also found that isolates H9, H11, H19, H23, H29, H32 and H36 were grown in TCBS agar medium and showed green colony. They were motile, Gram

Table 2. Salt tolerance test of *Vibrio* spp. isolates

Isolates name	Tests performed				
	Growth in 0% NaCl	Growth in 4% NaCl	Growth in 6% NaCl	Growth in 8% NaCl	Growth in 10% NaCl
H1	-	+	+	+	+
H2	-	+	+	+	+
H3	-	+	+	+	+
H4	-	+	+	+	+
H5	-	+	+	+	+
H6	-	+	+	+	+
H7	-	+	+	+	+
H8	-	+	+	+	+
H9	-	+	+	+	+
H10	-	+	+	+	+
H11	-	+	+	+	+
H12	-	+	+	+	+
H13	-	+	+	+	+
H15	-	+	+	+	+
H16	-	+	+	+	+
H17	-	+	+	+	+
H18	-	+	+	+	+
H19	-	+	+	+	+
H20	-	+	+	+	+
H21	-	+	+	+	+
H22	-	+	+	+	+
H23	-	+	+	+	+
H25	-	+	+	+	+
H26	-	+	+	+	+
H29	-	+	+	+	+
H30	-	+	+	+	+
H31	-	+	+	+	+
H32	-	+	+	+	+
H33	-	+	+	+	+
H34	-	+	+	+	+
H35	-	+	+	+	+
H36	-	+	+	+	+
H37	-	+	+	+	+

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negative, positive in oxidase test, positive in MR (except isolate H36 showed negative) and indole test (Table 01). These isolate did not grow in trypton broth without NaCl and gave negative result in dextrose utilization test, but showed positive in O-F test and catalase test. These isolates showed negative in citrate and urese test and positive in gelatinase test (Table 01) and identified as *Vibrio parahaemolyticus*. Furthermore, the isolates H5, H12, H18, H25 and H26 were grown in TCBS agar media and showed positive in luminescence and H_2S test. During the course of study it was found that, these isolates were Gram negative, motile, positive in O-F, MR and indole test and negative in VP test (Table 01). In this study it was found that these isolates were negative in dextrose fermentation test and citrate test (Table 01) and identified as *Vibrio harveyi*.

3.6 Antibiogram Study of *Vibrio* spp. Isolates

The bacterial isolates belong to *Vibrio spp*. was found to vary in their antibiotic sensitivity pattern to the eight antibiotic discs used. Three antibiotics were found 100% sensitive in this study. All of the isolates were susceptible to streptomycin, chloramphenicol and ciprofloxacin. Conversely majority of the isolates were resistant to co-trimethoxazole (41%), azithromycin (30%) and novobiocin (24%). The percentages of antibiotic susceptibility pattern were shown in (Figure 01). The zone of inhibition given by the antibiotics was varied with different isolates ranging from 14 mm to 40 mm in diameter (Table 3). Formation of clear zone by various antibiotic discs against *Vibrio* sp. Isolates were found.

Table 3. Antibiotic sensitivity pattern of *Vibrio* spp. isolates

Isolates name	Name of antibiotics with their sensitivity pattern (mm)							
	S	E	G	CH	CI	CO	AZI	NO
H1	34	22	16	36	32	34	30	22
H 2	30	29	28	32	28	33	24	24
H3	33	31	25	30	30	R	18	R
H4	27	22	30	33	18	33	14	16
H5	30	31	22	34	26	31	R	14
H6	32	R	31	28	32	R	26	17
H7	26	33	29	30	20	30	32	R
H8	31	R	R	36	24	R	24	26
H9	29	30	33	40	23	R	30	21
H10	33	24	30	38	27	28	R	R
H11	34	28	27	31	32	27	R	14
H12	29	30	18	33	30	R	29	18
H13	33	34	33	40	32	16	32	22
H15	25	R	R	31	26	23	R	R
H16	31	R	24	29	28	R	30	16
H17	30	25	16	33	30	33	R	22
H18	24	R	26	36	20	R	16	19
H19	27	30	32	30	24	25	R	R
H20	33	29	R	32	22	R	R	16
H21	24	33	30	34	32	R	32	14
H22	31	R	22	35	20	R	22	24
H23	28	31	24	38	28	26	R	22
H25	32	R	R	33	27	31	25	R
H26	34	27	29	28	32	R	28	R
H29	29	33	R	30	26	30	R	18
H30	31	29	33	34	23	R	27	20
H31	24	34	30	32	29	32	R	R
H32	32	R	26	30	31	R	30	23
H33	28	31	20	36	22	R	22	24
H34	26	25	30	30	30	32	20	R
H35	33	32	24	34	29	R	27	16
H36	25	30	29	30	31	26	30	18
H37	34	R	30	32	26	30	R	23

Notes: E = Erythromycin; CH = Chloramphenicol; S = Streptomycin; G = Gentamycin; CI = Ciprofloxacin; CO = Co-trimethoxazole; AZI = Azithromycin; NV = Novobiocin.

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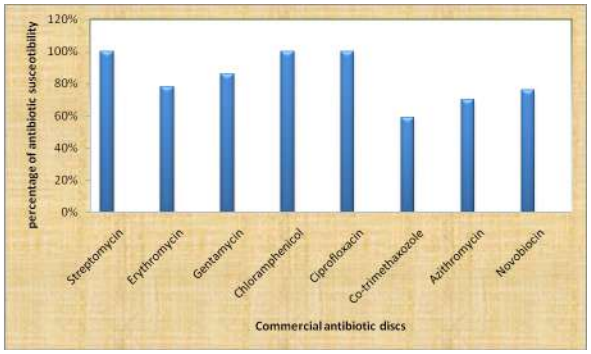

Figure 1. Percentage of susceptibility of *Vibrio* spp. isolates to commercial antibiotic discs

4. Discussion

All the isolates of the present study were found gram negative, while positive in motility test, catalase and oxidase. Most of the isolates were positive in MR and indole test but negative in VP test. These all findings are similar with the findings of Chakma *et al.* 2018 [9].

Again all isolates were positive in O-F test that means all isolates were capable of acid production but most of the isolates were unable to produce gas in dextrose fermentation test (except isolates H8, H10, H16,H17, H30 and H34). In the present study it was found that most of the isolates were negative in citrate utilization test as well as H_2S test, same result was found by Teng *et al.* 2017 [10]. Maximum isolates showed negative in lysine decarboxylase test and urease test, but showed positive result in gelatin liquefaction test. Similar biochemical results are reported by other researchers [11-16].

In this study, three types of *Vibrio* species such as *Vibrio Alginolyticus*, *Vibrio parahaemolyticus* and *Vibrio harveyi* were detected. Species were identified on the basis of different biochemical test which is similar with the test results for *Vibrio Alginolyticus* [12, 17]. *Vibrio parahaemolyticus* similar results were also obtained by Jayasree *et al.,* 2006 and Alagappan *et al.,* 2010 [18, 19] and according to the findings of Jiravanichpaisal *et al.,* 1994 and Lavila-Pitago, 1996 [20, 21] the same results were for their study of *V. harveyi*.

The prevalence uses of antimicrobial agents against the *Vibrio* sp. are described by various researchers over the last 20 years [22-26]. As far in the present study, 59% isolates were resistant to co-trimethaxozole, which is mostly similar to Razvykh *et al.,* 1990 [27]. According to Abraham *et al.,* 1997 [28], it is compared with the present study that 30% and 24% resistance were shown to azithromycin and novobiocin respectively (Table 03). The present study showed that 78% sensitive to erythromycin, but Thakur *et al.,* 2003 [29] reported 100% sensitivity. According to Herwig and Gray, 1997 [30] a mostly similar comparison found with the present study that gentamycin showed 82% sensitivity pattern.

The present antibiogram study showed that 100% sensitivity to ciprofloxacin, chloramphenicl and streptomycin. Karunasagar *et al.,* 1994 [31] also found 100% sensitivity to chloramphenicol. Erythromycin are frequently used in Bangladesh in doses similar with other countries [32], and these drugs are known to yield plasmid-mediated resistance in aquatic bacteria [33].

The tiger shrimp, *Penaeus monodon* is one of the most nutritional and economic important species of Bangladesh. However, pathogenic effects reduce the production of this species and *Vibrio* is one of the vital causal agents. It was observed in the present study that *Vibrio spp*. was occurred in the *Penaeus monodon* of shrimp pond in Satkira and three species of *Vibrio* like *Vibrio Alginolyticus*, *Vibrio parahaemolyticus* and *Vibrio harveyi* were observed. The present study has been emphasized on the antibiogram of *Vibrio* spp. and study found 100% susceptibility to streptomycin, chloramphenicol and ciprofloxacin.

5. Conclusion

The findings of the study suggest to focus on the further molecular test with a large number of fish samples. In addition, the host-pathogen interaction also need to be analyse with the exposure of environmental factors responsible for the outbreaks of the diseases caused by *Vibrio spp*., if any.

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Conflict of Interest

The authors declare no conflict of interest.

Authors Contribution

All authors are equally responsible for every task of regarding the manuscript.

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ARTICLE

Prevalence of Kudoa in Fish Fillets Caught in Para State

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ABSTRACT

Kudoa is a myxozoan that causes myoliquefaction in marine fishes. Most of species only affect fish, but a *K. septempunctata* outbreak was reported in 358 people. Although many species of *Kudoa* are known, none was described in *Brachyplatystoma filamentosum*, *Brachyplatystoma rousseauxii*, *Mugil curema*, *Plagioscion squamosissimus* or *Oxydoras niger* until now. Due to the economic cost of eliminating seafood presenting myxozoan lesions, this study aimed to describe lesions found at necropsy and histopathology, as well as to detect this myxozoan by molecular techniques. For this purpose, were sampled 85 fish of the following species: *Brachyplatystoma filamentosum*, *Brachyplatystoma rousseauxii*, *Mugil curema*, *Plagioscion squamosissimus*, and *Oxydoras niger* from Colares and Vigia, Pará, Brazil. Necropsies were carried out to describe lesions and molecular techniques (PCR and sequencing) were applied for identification. Although muscle lesions were not observed at necropsy, histopathology revealed bacterial colonies, coagulative necrosis, dystrophic calcification, eosinophils, hemorrhage, parasitic pseudocysts, protozoan, and vacuolization. After sequencing, *K. shiomitsui* (GENBANK: LC128646) was identified as the most similar causative agent of fishes infection, but due to phylogenetic results and identity we suggest that the myxozoan found could be a new specie. Also, high parasitism of this myxozoan was observed in fishes sampled, i.e., 90 % in Colares and 100% in Vigia.

1. Introduction

The genus *Kudoa* is a myxozoan of Multivalvulida order and Kudoidae family. Until now 63 species of this protozoan were described in fish species. This parasite is well described in the musculature of seafood, causing myoliquefaction or cyst formation [1], which can depreciate the product, leading to economic losses.

Kudoa was found in many species of fish and countries, such as *Thrysites atun* and *Beryx splendens* in Australia and South Africa [2], *Oncorhynchus kisutch* in Northwest Pacific of North America [3], *Merluccius productus* and

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Oncorhynchus kisutch in Canadian Pacific [3-5], *Salmo salar* in Washington Pacific [4], *Crypsilurus* and *Engraulis japonicas* [4], *Zeus faber* in Mauritania [4], and *Scomber sombrus L*. in North Sea [1].

In Brazil, it is described in the following hosts: *Aequidens plagiozonatus* [5], *Steliffer minor* [6], *Chaetobranchopsis orbicularis* [7], *Mugil liza* [8], *Mugil platanus* [9], *Scomberomorus brasiliensis* [10], *Odontesthes bonariensis*, and *Micropogonias furnieri* [11].

Due to no report and impact evaluation in the fish fillets with *Kudoa* from Para state, this study aimed to describe lesions by necropsy and histopathology, as well as to detect and identify this myxozoan by molecular techniques.

2. Materials AND Methods

2.1 Sampling

Eight five fishes of the following species were randomly sampled through net fishing: *Brachyplatystoma filamentosum*, *Brachyplatystoma rousseauxii*, *Mugil curema*, *Plagioscion squamosissimus*, and *Oxydoras niger* from Colares and Vigia, Pará, Brazil (Table 1).

Table 1. Number and species of fishes sampled in Colares and Vigia, Pará, Brazil

Specie	Colares	Vigia	Total
Brachyplatystoma filamentosum	10	12	**22**
Brachyplatystoma rousseauxii	10	10	**20**
Mugil curema	0	13	**13**
Oxydoras niger	10	10	**20**
Plagioscion squamosissimus	10	0	**10**
Total	**40**	**45**	**85**

2.2 Necropsy and Histopathology

After caught, fish necropsies were carried out according to Noga [12] to observe macroscopic lesions and sampling of the muscle fragments for histopathology due to the high occurrence of *Kudoa* in this tissue. For this purpose, a 1-cm³ of muscle portion was fixed in 10% neutral buffered formalin followed by processing using standard histological techniques and embedded in paraffin [12]. Hematoxylin and eosin was used for staining.

2.3 PCR and Sequencing

For PCR (Polymerase Chain Reaction), it was weighted 20 mg of muscle sampled during necropsy for each fish analyzed. Besides, it was added 500 µL of buffer (50 mM EDTA, 50 mM Tris, 150 mM NaCl, pH 8.0) into a tube with the muscle weighted. After this step, samples were frozen (-80ºC) and thawed (20ºC) for 4 hours. All this treatment of temperatures was performed 3 times. Then, the lysis was made with 50µL of proteinase K (10 mg mL) under 50°C for 4 hours. Finally, lysate was extracted with Wizard® SV Genomic DNA Purification System (PROMEGA®) kit, according to manufacturer´s recommendations.

The PCR was performed with the primers 18f (5' CAC-CAG-GTT-GAT-TCT-GCC 3') and 1492r (5' GGT-TAC-CTT-GTT-ACG-ACT-T 3') as described by Baker et al. [13]. Thereby, it was used Platinum®Taq DNA Polymerase (Invitrogen®) mix with the following concentrations: Buffer 1X, dNTP 0.2mM, $MgCl_2$ 1.5mM, primers (18f and 1492r) 0.2 µM each, and Platinum®Taq DNA Polymerase 1 unit. The reaction protocol was the following: denaturation under 95ºC for 5 minutes; followed by 40 cycles of 95ºC for 1 minute, 50ºC for 1 minute, 72ºC for 1 minute, and final extension of 72ºC for 5 minutes. The amplicon was visualized under ultraviolet light by eletrophoresis with agarose 1.5%.

The 1200 bp amplicons were purified with an Ilustra Microspin™ S-400 HR Columns Kit (GE Healthcare®) according to the manufacturer's instructions for identification by Sanger sequencing. For this, the purified amplicon was sequenced in both directions using BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems) on an Applied Biosystems capillary 3500 Genetic Analyzer. The quality of the electropherograms was assessed in Sequencing Analysis version 5.4 (Applied Biosystems). After this step, sequences were identified by similarity using BLAST (Basic Local Alignment Search Tool) algorithm.

A nucleotide sequence of approximately 1200 bp was used to query the GenBank library to arrive at the closest strain type and thus attain a species affiliation and possible identification to that level. To compare the sequences from different strains found in the GenBank library, the nucleotide sequences were aligned with ClustalW from MEGA software, version 7. For MEGA, the item "Find Best DNA/Protein Model" recommended the Maximum Likelihood method based on the General Time Reversible model with gamma distribution as model evolutionary rate differences among sites (2 categories, parameter = 1.3963) for our sequence as the most parameter-rich evolutionary model. We used codon positions included were 1st+2nd+3rd+Noncoding, 1935 positions in the final dataset, and bootstraps with 500 replicates.

The sequences used for phylogenetic tree were our sequence, *Kudoa amamiensis* (genbank: AY152748), *K. anatolia* (genbank: MH310914), *K. barracudai* (genbank: KU212177), *K. hexapunctata* (genbak: LC316999), *K. iwatai* (genbank: AB693041), *K. lateolabracis* (gen-

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bank: AY382606), *K. neothunni* (genbank: LC317001), *K. niluferi* (genbank: MH310915), *K. ogawai* (genbank: KX163082), *K. ovivora* (genbank: AY152750), *K. puraishii* (genbank: KF413764), *K. rayformis* (genbank: KR140014), *K. shiomitsui* (genbank: LC128646), *K. septempunctata* (genbank: AB693040), *Kudoa* sp. (genbank: AY302723), *K. thalassomi* (genbank: LC382036), *K. thyrsites* (genbank: AY382607), and *Pseudopolystoma dendriticum* (genbank: FM992707).

2.4 Statistical Analysis

The prevalence of *K. shiomitsui* detected by PCR and evaluation of risk factors were calculated for each place of sampling, also for sex and host specie. The comparison between factors cited was performed with Fisher´s exact test with confidence interval of 95%. This statistical analysis was performed and visualized in GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

3. Results

During necropsy, it was observed lesions in the liver, kidney, spleen, and stomach, such as hemorrhage, increase in the size of the organ, and necrosis. Despite no muscle injury was observed, bacterium colonies, coagulative necrosis, dystrophic calcification, eosinophils, hemorrhage, parasites pseudocysts, protozoan and vacuolization were confirmed by histopathology (Figure 1). The most common alterations were coagulative necrosis, hemorrhage, and protozoan presence (Figure 2).

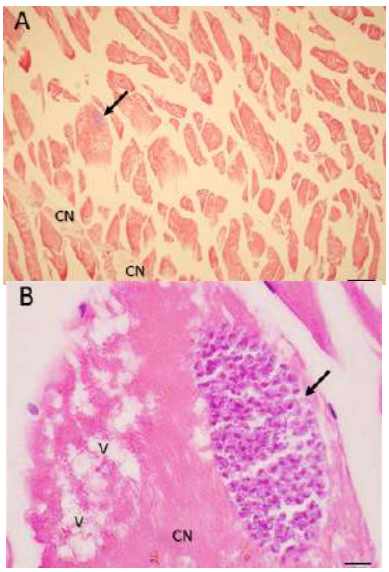

Figure 1. Muscle histopathology. A: Observation of coagulative necrosis (CN) and parasite cyst (arrow). B: Visualization of coagulative necrosis (CN), parasite cyst (arrow), and vacuolization (V). HE staining

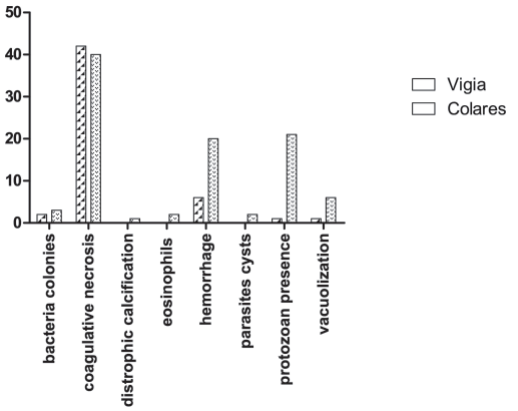

Figure 2. Prevalence of microscopic lesions in the muscle of fish analyzed in Vigia and Colares municipality

The PCR amplified a product of 1200 bp, which presented 86% of identity with *Kudoa shiomitsui* (GENBANK: LC128646), confirming that the myxozoan observed in histopathology. Analyzing phylogeny results (Figure 3), we observed that our sequence grouped with some species of *Kudoa*, such as *K. shiomitsui*, *K. ogawai*, *K. rayformis*, *K. thyrsites*. As showed Figure 3, our sequence formed another clade, which suggests that the myxozoan detected in our study could be a new species.

Figure 3. Molecular Phylogenetic analysis by Maximum Likelihood method based on the General Time Reversible model with gamma distribution as model evolutionary rate differences among sites (2 categories, parameter = 1.3963). The tree with the highest log likelihood (-14660.88) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Bootstrap with 500 replicates

The species analyzed in this study presented highly parasitism, since 70% to 100% (Table 2). Thereby, *Branchyplastoma* filamentosum, *B. rousseauxi*, and *Oxydoras niger* presented 100% of parasitism, *Mugil curema* with 92,30% and *Plagioscion squamosissimus* with 70%.

Afterwards, it was also observed that 97.77% of fishes sampled in Vigia and 92.50% in Colares (Table 2) were positive for this myxozoan, which was statistically significant ($p < 0.05$) by Fisher´s exact test. On the other hand, when gender was evaluated as a factor for this disease, no statistically significant differences were observed ($p = 0.4368$), the opposite for host species ($p < 0.05$), i.e., only place and host species were identified as risk factor for this parasitosis.

Table 2. Prevalence of *Kudoa shiomitsui* in fishes sampled in Colares and Vigia, Pará, Brazil

Specie	Colares	Vigia	Total
Brachyplatystoma filamentosum	10 (100%)	12 (100%)	**22**
Brachyplatystoma rousseauxii	10 (100%)	10 (100%)	**20**
Mugil curema	*	12 (92,30%)	**13**
Oxydoras niger	10 (100%)	10 (100%)	**20**
Plagioscion squamosissimus	7 (70%)	*	**10**
Total	**37 (92,50%)**	**44 (97,77%)**	**85 (100%)**

Note: * Fish species that were not sampled.

4. Discussion

Although *Kudoa* species cause myoliquefaction in fish muscle, Tsuyuki et al. [14] reported that proteolytic activity seems to be linked to the stage of parasitic infection, pH value, as well as to the level of infection [15]. This information explains why the present study found lesions only at microscopy.

During infection, the parasite destroys the sarcolemma, which lead to the development of a fibroblast layer around it and development of pseudocyst with black appearance. Although, in some species of fish there is no formation of black pseudocyst and in the macroscopy the muscle is intact [16], such as *Scomberomorus braziliensis* [10], which also could be the case of the fishes sampled in this study.

In Brazil, there were reported *Kudoa* in *Lutjanu analis, L. jocu, Bagre marinus, Aspitor luniscutis, Scomberomorus braziliensis* [10], and others species, but the fish species studied in this research has no report of myxozoan. According to Eiras et al. [11] investigation of *Kudoa* in fish along the Brazilian coast is very scarce, which supports the importance of this study due to economic loss that this protozoan can cause in seafood.

Although we observed that only locality is a risk factor for the parasitism, St-Hilaire et al. [6] and Levsen et al. [4] also described that age and size of fishes could lead to higher parasitism, being the larger (>600 g) and sexually mature more susceptible. This is an important point since the fishes caught normally are adults, increasing the risk of parasitism.

The diagnosis of *Kudoa* in fish fillets that not present myoliquefaction is difficult in sanitary inspection by observation of the muscle. For this reason, this study showed that histopathology and PCR help and increase detection of this myxozoan. Shaw et al. [17] also reported that even when visualization and histopathology are negative for this parasite, PCR detected *Kudoa*. Due to the sensitivity, specificity, cost, and time spent, molecular techniques can be used for surveillance and diagnosis of this protozoan in fish fillets.

Although the main problem of *Kudoa* parasitism is associated to myoliquefaction in fish fillets, the species *K. septempunctata* were responsible of a 358 people outbreak, after consumption of *Paralichthys olivaceus* [16], showing the zoonotic potential of this group of parasites.

Other studies also show samples with detection of *Kudoa shiomitsui*, such as Kasai et al. [18], which reported this specie of myxozoan in fish samples collected in different markets in Japan from the Inland sea and the west, the east China sea and the Pacific Ocean in the period of July 2013 to December 2015. In the study, gills and viscera were collected and examined, then the presence of myxozoan of the genus *Kudoa* was observed through a dissection microscope confirmed by PCR. From the 75 analyzed samples of monocantid fish, 8 presented four different species of *Kudoa*, being in two fishes *K. septempuctata*, in other three *K. thyrsites* and *K. shiomitsui*, located in the pericardium with the presence of more than 80 cysts.

In another study, two sampling boxes containing 25 fish with three different species of *Merluccius*. They found parasites in nodules and cysts that were observed through a dissection microscope and DNA sequenced. As a result 89% presented the presence of different species of *Kudoa* [19].

5. Conclusions

With this study we conclude that *Brachyplatystoma filamentosum*, *Brachyplatystoma rousseauxii*, *Mugil curema*, *Plagioscion squamosissimus*, and *Oxydoras niger* were highly parasite by *Kudoa*. It was also observed that municipality and host species could increase the risk of this infection. With this, is crucial surveillance of this parasite in those fishes to decrease the economic loss and guarantee food safety.

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Conflict of Interest

For this research, there are no conflict of interest.

Availability of Data and Material

All samples and DNA are deposited in Biotechnology Institute of São Paulo State University.

Code Availability

Not applicable.

Author's Contribution

Marianna Vaz Rodrigues: the author contributed in sampling, as also performing necropsy, histopathology, molecular techniques and write of manuscript.

Patrícia Tidori Miura: the author contributed in molecular techniques and write of manuscript.

Jéssica Fernandes de Oliveira: the author contributed in molecular techniques and write of manuscript.

Maria das Dores Correia Palha: the author contributed in sampling, necropsy and write of manuscript.

João Pessoa Araújo Júnior: the author contributed in molecular techniques and write of manuscript.

Ethics Approval

Due to the study used fish caught and marketed in Colares and Vigia, there were no need for ethics approval.

Consent to Participate

All authors agree to participate of this study.

Consent for Publication

All authors agree in publication this study.

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ARTICLE

Portrayal of Camel Production in The Desert Ecosystem of Pakistan

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Pakistan

ABSTRACT

Camel plays a pivotal role in the subsistence pastoral economy of diverse ecozones extending from Gobi Desert and India in central Asia to Somalia and Ethiopia in the horn of Africa. Camel has special attributes including its appearance and ability to survive in hot, harsh and versatile arid environments. Camel has fascinated mankind as it can tolerate many stresses like heat; scarcity of water; water with high salinity and shortage of feed. Camel can digest dry matter and coarse crude fiber better than any other ruminants. Among domestic animals, the dromedary is most important animal being survive in hot, arid and semi-arid regions and has potential to produce higher quality foods (meat and milk) under extreme environments at lower costs. Camel can tolerate solar radiations, higher temperatures and water scarcity. Camel consume those feed materials which remain un-utilized by other domestic animals, thus thrive well on sandy deserts with poor vegetation. Adaptation of Camelids in Pakistan is very well to their native environment as they are performing and well sustaining a life in hostile deserts. The dromedaries provide milk and meat to the pastorals and herders in those areas where the survival of other livestock species is very tough. So, camels equilibrate the food security chain in the deep deserts and provide nourishment to its keepers; proving it to be a good candidate of food security and sovereignty in the desert ecosystem.

1. Introduction

The potential of camel is well known as it provides meat, milk, hairs, wool and transportation to many people in the world. Millions of people are getting benefits from camels in different ecozones. It is the animal of arid, semi-arid, mountainous and especially desert areas where the survival and performance of other livestock species seems difficult [1]. Unique physiological characteristics aid the camel for this environment. It has food reservoir in the form of hump deposit on its back which stores energy in terms of fat. The fatty hump provides energy during the drought conditions when there is shortage of food and water. Camel hump doesn't contain water as most of the people think; rather it has stored fat by which the camel can live off in the food scarcity period [2].

Camel - an even toed ungulate has special defense system as it is fast runner and can go easily on sand, bite and spit on approaching when threatened by people [3]. Camel is versatile in its properties as it sustains life in adverse environment, eat and drink less, while no match regarding performance [4]. The popular notion of "ship of

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the desert" about camel is now getting changed to a "food security animal". Mainly two types of camels are found in the world; dromedary (*Camelus dromedarius*) with one hump, mostly found in Arabian deserts, Afghanistan, Iran, Central and South Asia [5]. The dromedary term is derived from "*dromos*" which is a Greek word used for road and applicable to racing and riding camel. The second is Bactrian (*Camelus bactrianus*); having two humps, from an area found in Central Asia, China and Russia while the name derived from a place "*Baktria*" on the river Oxus in northern Afghanistan from where it is considered to be originated [6].

2. Camel's Population Distribution in Pakistan

Worldwide its population is 35 million and Pakistan ranks 8th among major camel raising countries [7] having 1.1 million camels [8]. Camels are found in the rangeland of Balochistan, coastal and deserted areas of Cholistan, Thal and Tharparker. In Pakistan, regarding province wise distribution of camels, the Balochistan has highest population of dromedaries as about 41%, Sindh has 30%, Punjab has 22% while Khyber Pakhtun Khwa (KPK) has 7% [9] (Figure 1). Pakistan has maximum population of dromedaries while few herds of Bactrian are also present in the northern areas. There are four ecological zones of camel production; viz: 1- sandy deserts including Thal & Cholistan in Punjab while Thar in Sindh; 2- Costal mangroves including Badin, Thatta, and Karachi districts of Sindh; 3- Mountainous tracts of Balochistan, Dera Ghazi Khan of Punjab and Dera Ismail Khan of KPK; 4- Irrigated plains including all irrigated districts of Punjab and Sindh [10].

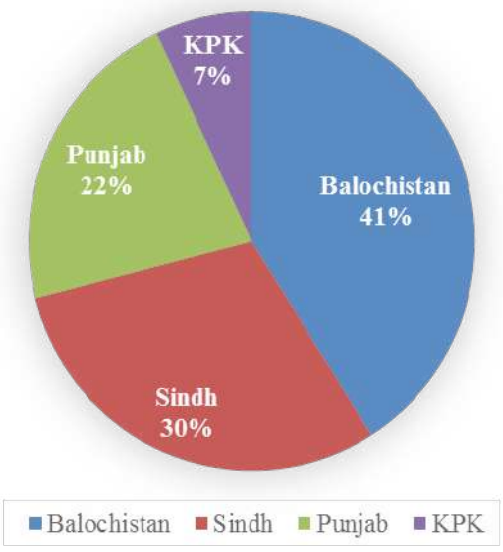

Figure 1. Quantitative distribution (%) of Pakistan whole camel population

3. Camels in Pakistan

Camels in Pakistan are performing very well in their native environment, in hot and harsh deserts. In the areas which have Extreme environmental conditions affecting the performance of other animals, the dromedaries proving its worth quite a nice way [11]. The unique attributes camel has gifted from God; enable him to thrive best in rough topography, poor vegetation, water scarcity and solar radiations [12].

Two types of camels are being found in Pakistan; 1- Mountainous camels which are also known as "Pahari" mostly found in north parts of Punjab and Balochistan. 2- Riverine camels native to the irrigated plains of Punjab and Sindh and commonly found in deserts. Up till now there are twenty breeds of camel documented in Pakistan [10] (Table-1).

Table 1. Camel Breeds in four provinces of Pakistan

Province	Camel Breeds
Punjab	Marecha (Mahra), Barela (Thalochi), Bagri (Booja), Mountainous (Cambelpuri) and Kalachitta
Balochistan	Brahvi, Kharani, Kachhi, Makrani, Lassi, Pishin and Rodbari
Sindh	Dhatti, Larri (Sindhi), Kharai, and Sakrai
KPK	Ghulmani, Gaddi, Khader and Maya

4. Camel Production Systems

There are three major camel production systems found in Pakistan; (1) Nomadic production system, (2) Transhumant production system, (3) Sedentary production system. These all production systems are associated with climatic conditions, plant phonology, topography and water availability; thus, clearly depicts the socio-economic importance of the camels in Pakistan. In Nomadic production system the camel rearing is mainly linked with the social life of the pastorals and camel herders. The nomads are constantly moving from place to place due to the reason of lack of forage for grazing/browsing of camels and the water shortage problems. They locate and found the place where the feed is available for some time and by vanishing the resource they move to next place. Almost 26% of the pastorals/camel herders follow this system in Pakistan. This production system is characterized by three basic features; 1- Seasonal and disaster migrations are obvious with main objective of survival, 2- Camel herds are diversified with sheep, goat and donkeys, 3- Sharing and loaning of camel herds are the routine activities practiced by these herders/nomads [10].

In transhumant production system there is shifting of the tillage operations in rain fed area during the rainy

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seasons. Here in this system, the fundamental objective of availability of feed and water cause the migration of the herders. There are 23% camel herders which are involved in this system. Rest of the 50% camel herders are involved in sedentary production system which constitutes the major proportion of the household income. Women play major role in this system; they are not only involved in camel rearing but also play their important role in the value addition of byproducts, convert them into useful products, market them and put major share in home economy [10-11].

5. Milk Production

Production in the traditional system is mainly geared to milk production. Male calves face a stiffer competition with the herders for their dam's milk than female calves. They are often allowed to one teat only or given access to dam's udder after milking. As a consequence pre-weaning survival is less and lower weaning weights are achieved. The basic factors in the growth of camel calves are availability of milk from dam and skilled management of calves. There is a severe competition between camel calves and farmer's family regarding the availability of milk. Male calves deprived of their due share of milk, that exhibit detrimental effects on their growth, leading to a downward trend in their meat production potential [12]. On the other hand, milk off take from dam with male calf is 80% and from dam with female calf is 30%, respectively. Approximately, 55% of the total milk production of camels is taken by the calf [5]. By minimizing this percentage camel milk yield can be increased. Weaning of calf could be the possible solution for these problems [12].

Dairy potential of Pakistani dromedary camel is well known and these are exported to Gulf States for dairy purposes. Reported average lactation yield of Marecha she-camel is 4179 liters per year which is probably the best milk yielder in the world having longer lactation period as 270 to 540 days while its total milk yield ranges from 1300-4200 liters (Figure 2). Barela is also a best milk producing breed having prominent milk vein. In desert conditions and in the area of poor fodder production, its reported average yield is 8-12 liters per day. Some heavy breed camels produce up to 35 liters milk per day [13]. Author of present study has visually observed some specimen of Marecha camel in Thal Desert producing up to 25-35 liters milk per day. Camel can maintain its average milk yield for a long time (12-18 months, at least for one year) by the provision of adequate feed and water which is not possible in other domestic species. Frequency of milking is more in camels (up to 5-6 times a day). It is said that whenever a man needs milk from camel; he has to tie the camel legs and she will be ready for milking. Camel milk is rich source of vitamin C, while concentration of protein, fat, minerals and vitamins is almost same like cow milk [14-16]. Camel milk is superior to the milk of other domestic species as it is rich in phosphorus concentrations. Camel milk has higher protective protein contents which perform an inhibitory action against certain bacteria thus has increased shelf life. It is easy to market camel milk with basic hygienic conditions even in the higher temperature [13].

Figure 2. Marecha (Mahra) camels of Punjab, Pakistan

Figure 3. Barela (Thalochi) camel of Punjab, Pakistan

Camel farming could be practiced as commercial dairy farming in the areas of Thal and Thar Deserts and Cholistan rangelands. Camel milk is consumed as such and also with some value additions in Pakistan. Tea, yogurt, lassi (whey proteins) are also made from camel milk. Camel milk has different sensory characteristics. Pakistani camels qualify to be a good dairy animal as it has all prominent dairy features. By the adoption of modern husbandry practices; camel could be the valuable source for future food production especially in arid, semi-arid, mountainous and deserted areas. Average milk production per lactation of Pakistani camel is reported as 2920 kg with per day yield of 8 kg in extensive conditions [10] (Table 2).

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Ice cream

Chocolate

Cheese

Yogurt

Flavoured milk

Figure 4. Camel milk by-products

Table 2. Camel milk production

Country	Milk yield (kg)	Lactation length (months)	Average milk yield (kg)
Pakistan	2920	16-18	8
India	2482	18	6.8
Somalia	1825	9-18	5
Tunisia	1460	9-16	4
Algeria	1460	9-16	4
Ethiopia	1825	12-18	5

6. Growth Rate

The growth (daily weight gain) of camel calves is excellent as other livestock species [17-19]. In provision of camel meat, mostly the old and spent camels are slaughtered in Pakistan. A very few numbers of castrated camels are used for fattening purpose. Camel is also slaughtered blissfully at Eid-ul-Azha by Muslims and that camels fetch a good price. Export opportunities are there, camel meat could be exported to Saudi Arabia, Gulf States, Egypt and Libya. In Pakistan 100-200 camels are slaughtered daily in different slaughter houses at meat day. Meat of camel is consumed as fresh, in minced form and also in barbeques and sausages. The taste of camel meat is very similar to beef. The amount of minerals, protein and ash is the same as that of beef, but camel meat contains less lipids (1.2-1.8% versus 4.0-8%) and high water contents (5-8% more) than that of beef. Dressing percentage of camel ranges from 45-55% (exceptionally 60% in some animals). Average slaughter weight is 400-660 kg and growth rate are 0.3-1.0 kg from birth to 1 year of age [2].

7. Camel by-products

Hair production is another valuable aspect of camel production; an adult and mature camel can produce 1 to 3 kg hairs per annum which are used for different purposes like making of ropes, mats, bags, blankets and carpets. Its first shorn especially in new born calves produces some fine wool which is used in blankets industry [2]. Saddles and shoes are made from its hides. The usual price of its hide is PKR 2000 to 5000. Camel plays an indispensible role in the socio-economics of people in arid, semi-arid and marginal regions of the world. Camel has a significant contribution to the livelihood of pastoral community which doesn't have any alternate mode of production system. Despite of the fact; the camel has been remaining a neglected specie by the scientists/development workers and very few attempts have been made so far to characterize its production potential and related parameters under natural habitat. While in extensive/traditional management system the camel production traits are very

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low so the traditional camel husbandry has no future [17-18]. Camel husbandry system is in a state of constant flux as the pastoralists are deviating/shifting from their traditional management system to semi-intensive and intensive management system. This rapidly changing scenario needs overall evaluation and there is an urgent need to undertake multi-disciplinary studies [19-21]. Recently the intensive studies have been taken in Pakistan regarding the growth performance of camel calves in different management systems and the reported range of average daily gain in Marecha camel calves is 0.4 to 1 kg, so it proves camel a good candidate for feedlot as well [22-23].

Lamps

Bags

Shoes

Hides, Leather

Figure 5. Camel by-products

8. Conclusions

Growing needs and emerging awareness has changed the notion of "Ship of the desert" to "food security animal" - Pakistan is not exception to this. Camel is of prime position in this regard as it meets the milk and meat demands of pastorals and people of arid, semi-arid and deserted areas. Camels in Pakistan are very productive and the potential in terms of milk and meat is well recognized now. But unfortunately, a little importance has been given to camel by the policy makers, scientists, development workers and coworkers. Is it a useless and unproductive animal, if it is so, its population would be diminished gradually but it's the other way round. Pakistan has a sizeable population and the dairy camels are exported to the Gulf countries for milk production then why these can't be harvested in Pakistan. Resurgence of special interest has to be given in this regard. We have to evaluate the production potential of the Pakistani camels and to build a country's data base for future studies in terms of production. Author of current study with his co-workers have evaluated and validated the results about the meat and milk production of camels in Pakistan under various management systems, which will show up the facts about the production potential of Camelids in Pakistan. This will pave a way for further investigations in camel science and aid to the overall scenario.

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ARTICLE

Mathematical Model to Estimate Carbon Footprint for EEG Incubation

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ABSTRACT

This work presents a performance comparison between several incubators models including CO_2 and NH_4 emission. A mathematical model for incubators carbon foot print was developed to estimate CO_2 and NH_4 emission. The program written by C^{++} language including convert line. The modular structure of program consists of a main programme and series of independent subroutine: each one deals with a specific parameter of the required data. The computer programme has a wide range of applicability several values of size of the machine (NO. egg), Fertility (F), Heat production embryo (HP_e), maximum CO2 level $(CO_2)m$, CO_2 level incoming air $(CO_2)_I$,RQ value (RQ) to estimate Heat production (HP), CO_2 production, Ventilation (V), Ventilation of egg (V_{egg}) Input data: Enter size of the machine, Fertility (F), Heat production embryo (HP_e), maximum CO_2 level $(CO_2)_m$, CO_2 level incoming air $(CO_2)_I$, RQ value (RQ) the results As the growth period passed from the first day of the twenty-first day, the amount of heat produced increased from 0.0001 to 0.35 w / egg, and ventilation from 0 to 352 m^3 / hr as well as the amount of carbon dioxide produced from 0.0000158 to 0.04318 lit/hr/Mach. As the number of eggs increased from 5,000 to 30,000 eggs, each of the heat produced increased from 923.4 to 5540.4 kg / hr, the resulting carbon dioxide from 32 to 190 lit / hr / Mach, and ventilation from 9 to 54 m^3/hr

1. Introduction

During these last decades, the production of poultry meat increased almost 108% from 54 to 112 million tons, corresponding to a 36% growth of its share in total meat production. Incubators need to maximize chick production, and this entails not only the incubation of more fertile eggs. Today, incubators need to achieve high production efficiency in a sustainable manner, which, in our view, includes maximizing the hatchability of healthy chicks with high survival rates and reducing carbon emissions from increased.

Carbon footprint (CFP) named Carbon profile - is the overall amount of carbon dioxide (CO2) and other greenhouse gas (GHG) emissions (e.g. methane, nitrous oxide, etc.) associated with a product. The carbon footprint is a sub-set of the data covered by a more complete Life Cycle Assessment (LCA) [6].

The non-ruminant sector is a minor N2O emissions contributor compared with ruminant N2O emissions. The poultry industry is the largest direct N2O producer of the non-ruminant livestock industries, contributing 92.8% of

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the total non-ruminant N2O emissions [8].

Oxygen (O2) is essential for respiration in birds. It is inhaled and carbon dioxide (CO2) is a product of respiration in birds ,For optimum poultry production, the concentration of carbon dioxide must not exceed 2500 ppm , also said that low concentration of hydrogen supplied (H2S) is fatal to poultry and human health , and reported that if the temperature remains within the range of 25ºC to 30ºC, air velocity of 0.1 m/s to 0.2 m/s can be maintained, but if the temperature goes beyond that an increase in air velocity will help aid convectional cooling. Furthermore, air velocity of 0.1 m/s to 0.2 m/s the movement pattern of air can be easily controlled through building design and ventilation within the building [5].

During the 21 days it takes to incubate the chicken eggs to hatch, the developing embryo requires different levels of CO2 at specific developmental stages. Gas exchange is closely related to respiration function [7].

The ammonia emission rates averaged 19.7 and 18.1 mg.h-1 per bird in the summer and winter, respectively, and increased with indoor temperature (r2 = 0.51 in summer; r2 = 0.42 in winter). Emissions are mainly produced at the end of the summer cycle, whereas in winter, their production begins earlier in the cycle. Also he found CH4 emissions of 0.44 and 1.87 mg.h-1 per bird in summer and winter, respectively. Also found in a study that the average nitrous oxide emissions were 1.74 and 2.13 mg.h-1 per bird in summer and winter, respectively [1].

The a direct relationship between NH3 emissions and indoor temperature was also observed. Although indoor temperature was identified as main variable influencing NH3 emissions, other variables, such as ventilation rate and bird activity, may also be influencing those emissions [10].

The authors suggested paying greater attention to air circulation in incubators and decreasing the temperature during the second stage of incubation for heavier eggs. It is known that during the incubation period, as the embryo develops, oxygen (O2) consumption and carbon dioxide (CO2) production increase. Therefore, the levels of CO2 and O2 in the incubator are also crucial factors for embryonic development and may affect performance both at hatching as well as post-incubation [3].

The different concentrations of CO2 applied in different stages of embryo development depress hatchability and that a concentration higher than 1% increased gradually in the first 10 days of incubation enhanced embryo growth, and improved hatchability. However, when applied after the 10th day of incubation it had no effect on hatchability [9].

Many studies focused on CO2 levels during the early and middle stages of incubation. Results showed that CO2 concentrations over 1% during the first 4 to 8 days of incubation, up to 4% from 10 to 18 days of incubation and more than 6% between day 9 and d 12 of incubation decelerated embryonic growth, increased late mortality and depressed hatchability [2].

It is known that retention of CO2 in the body due to impaired respiratory function, leads to an elevation of body fluid PCO2 and results in respiratory acidosis or primary hypercapnia. The removal of extra CO2 during incubation is therefore thought to be one of the vital functions of good ventilation. The normal CO2 level is around 0.1-0.5% [4].

2. Problem

Increase the concentrations of carbon dioxide and ammonia in incubation. Reducing growth rates due to increased mortality rates in chickens and consequently lower egg production rates.

3. Materials and Methods

Experiment was carried out through 2019 in an incubator factory at a privet closed system egg production farms in Gamasa, Governorate of Dakahlia, Egypt. To estimated carbon footprint and other greenhouse gas (GHG) emissions. Also study the factors affecting the increase of greenhouse gas emissions in incubator.

The incubator has a capacity of 5,000 eggs, (Fig. 1) spent 21 days, and we expect what will happen in incubator with a capacity of 20,000 and 30,000 eggs.

Incubator dimensions were (173 * 117 * 172 cm) had a nominal capacity of 5000 hens in production period, in Table 2.

Table 1. Cobb characteristics

Breeder	Performance
Age at depletion	65 weeks
Age at 5% production	24 weeks
Total eggs / hen housed	181.3 %
Hatching eggs / hen housed	176.3 %
Peak hatchability	90 %
Cumulative hatchability	85.6 %
Broiler chicks / hen housed	150.9 %
Livability from 24 weeks	92.3 %

Table 2. Technical specifications for incubation

The size of the incubation	173 * 117 * 172 cm
Wight	220 Kg
Egg Capacity	5280 egg
Range of The temperature	5 - 50 °C
Hatching rate	≤ 96%

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Precise temperature control	0 , ±1 °C
Operating voltage	AC220V-240V, 50Hz
Relative humidity	Less than 85%
Ambient temperature	10°C - 40°C
Electricity	800 Watt
Work forever	10 - 12 years

Figure 1. Incubation capacity of 5000 eggs

A mathematical model for incubators carbon foot print was developed to estimate CO_2 and NH_4 emission. The program written by C++ language including convert line. The modular structure of programm consists of a main programme and series of independent subroutine: each one deals with a specific parameter of the required data from image.

The computer programme has a wide range of applicability several values of size of the machine (NO. egg), Fertility (F), Heat production embryo (HPe), maximum CO2 level (CO2)m , CO2 level incoming air (CO2)I ,RQ value (RQ) to estimate Heat production (HP(, CO2 production , Ventilation (V) , Ventilation of egg (Vegg)

3.1 Input Data

Enter size of the machine, Fertility (F), Heat production embryo (HPe), maximum CO2 level (CO2)m , CO2 level incoming air (CO2)I ,RQ value (RQ)

3.2 Calculate Data

HP (kj/hr)=HPe (watt) × (F (%))/100 × (3600/1000) × size of the machine (egg)

CO2/O2 production (lit/kj) = 16.2 / (RQ Value) + 5

CO2 production (lit/hr/mach) = (HP (kj/hr))/(CO2/O2 production (lit/kj))

V (m3/hr)=(CO2 production ×1000) /((CO2)m - (CO2) i)

Vegg (m3/hr)=([size of the machine (egg) × (F / 100) × (3600 / 1000) × HP (W/egg)] / (CO2 / O2 production) (lit/kj)) / ((CO2)m - (CO2)i) ×1000

3.3 Output Data

Heat production (HP(, CO2 / O2 production , CO2 production , Ventilation (V) , Ventilation of egg (Vegg).

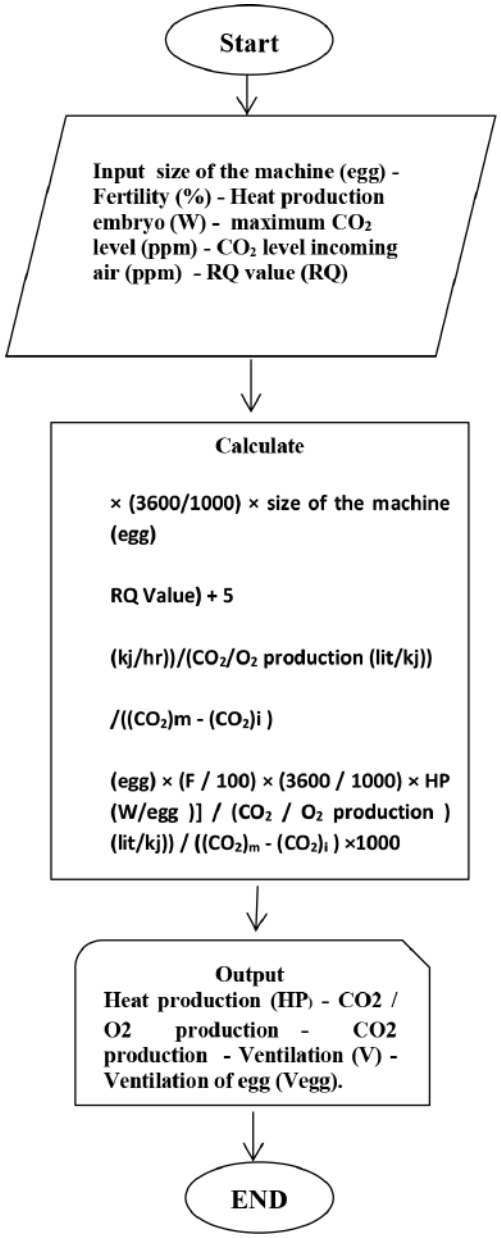

Figure 1. showing the inputs and outputs

4. Results and Discussion

4.1 The Effect of the Change in Egg Growth in Days on the Amount of Heat Produced

As the growth period passed from the first day of the twenty first day, the amount of heat produced increased linearly from 0.0001 to 0.35 W/egg (Fig. 2). A linear relationship was obtained between the the growth period and the amount of heat produced.

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$y = 0.0136x-0.0666$ $R^2 = 0.8077$

4.2 The Effect of the Change in Egg Growth in Days on the Ventilation

As the growth period passed from the first day of the twenty first day, the Ventilation increased linearly from 0 to 352 m3/hr (Fig. 3). A linear relationship was obtained between the the growth period and the Ventilation.

$y = 6.8196x-33.473$ $R^2 = 0.8077$

4.3 The Effect of the Change in Egg Growth in Days on the Amount of co_2 Produced

As the growth period passed from the first day of the twenty first day, the amount of co2 produced increased linearly from 0.0000158 to 0.04318 Lit/hr/mach (Fig. 4). A linear relationship was obtained between the the growth period and the amount of co2 produced.

$y = 0.0017x-0.0082$ $R^2 = 0.8077$

4.4 The Effect of the Change in the Number of Eggs on the Amount of Heat Produced

The number of eggs linearly increased from 5,000 to 30,000 eggs with increased heat produced from 923.4 to 5540.4 KJ/hr (Fig. 5). A linear relationship was obtained between the number of eggs and the amount of heat produced

$y = 0.1847x + 0.2$ $R^2 = 1$

4.5 The Effect of the Change in the Number of Eggs on the Amount of Co_2 Produced

The number of eggs linearly increased from 5,000 to 30,000 eggs with increased co_2 produced from 32 to 190 Lit/hr/mach (Fig. 6). A linear relationship was obtained between the number of eggs and the amount of co_2 produced

$y = 0.0063x + 0.1333$ $R^2 = 1$

4.6 The Effect of the Change in the Number of Eggs on the Ventilation

The number of eggs linearly increased from 5,000 to 30,000 eggs with increased Ventilation from 9 to 54 m3/hr (Fig. 7). A linear relationship was obtained between the number of eggs and the Ventilation.

$y = 0.0018x$ $R^2 = 1$

5. Conclusions

As the growth period passed from the first day of the twenty-first day, the amount of heat produced increased from 0.0001 to 0.35 w / egg , and ventilation from 0 to 352 m3 / hr as well as the amount of carbon dioxide produced from 0.0000158 to 0.04318 lit/hr/mach . As the number of eggs increased from 5,000 to 30,000 eggs, each of the heat produced increased from 923.4 to 5540.4 kg / hr , the resulting carbon dioxide from 32 to 190 lit / hr / mach , and ventilation from 9 to 54 m3/hr

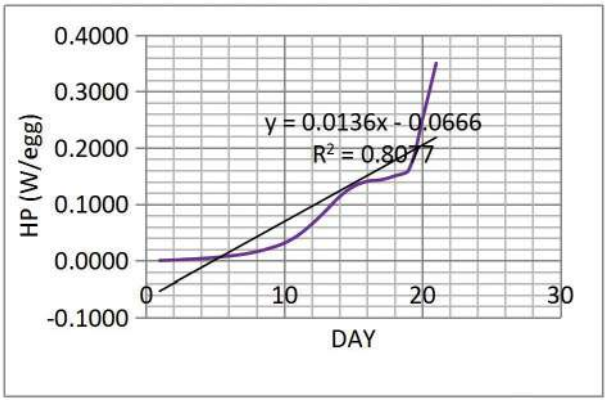

Figure 2. Relationship between day and Heat production

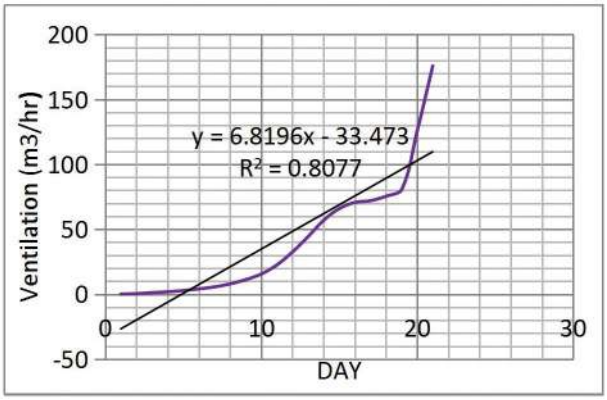

Figure 3. Relationship between day and ventilation

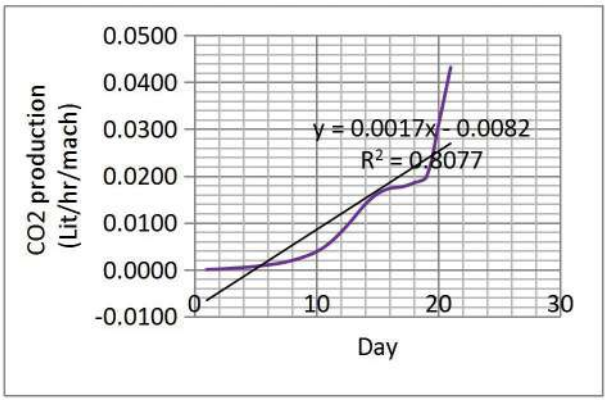

Figure 4. Relationship between day and CO_2 production

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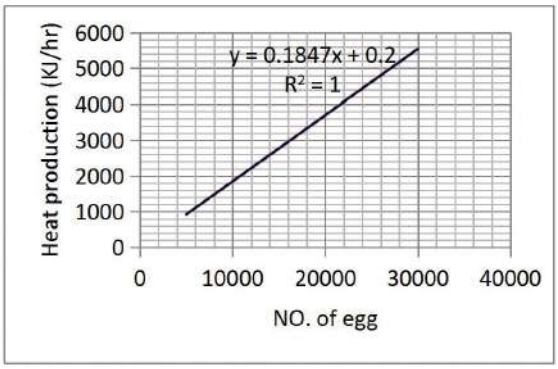

Figure 5. Relationship between number of eggs and Heat production

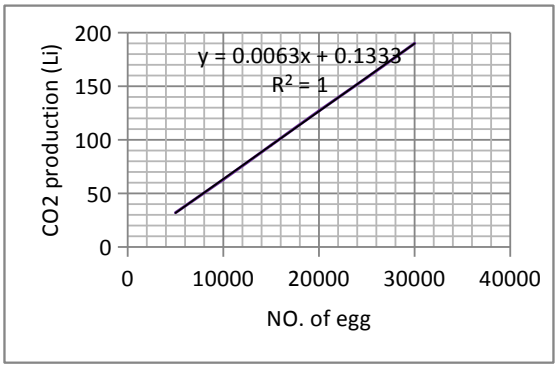

Figure 6. Relationship between number of eggs and CO_2 production

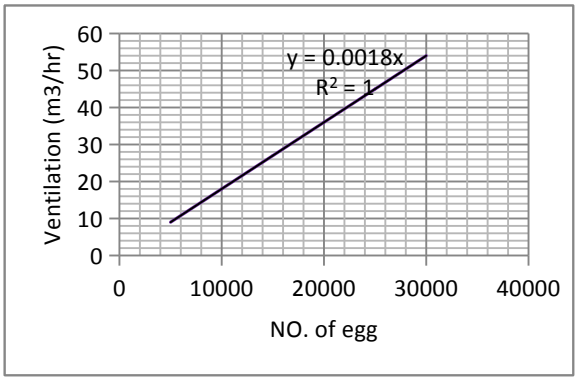

Figure 7. Relationship between number of eggs and ventilation

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ARTICLE

Butterfly Diversity on a Southeast Florida Military Base Located within an Urban Matrix

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ABSTRACT

South Florida is a renowned 'hotspot' for rare and endemic taxa, with insects and plants found in few other ecosystems. Specialized species evolved in Florida's stochastic climate, adapting to seasonal drought and flooding, hurricanes and high-wind tropical storms. As human population growth and development increased, and natural ecosystems disappeared, or became increasingly degraded, at-risk taxa now face additional threats, such as urban pesticide use and fragmented remnant habitats. The ability of species to adapt to these changing ecological factors is one of the dynamics that either impacts their fitness to greater survival or drives extirpation or extinction. Butterflies are native indicator species that can be used to document environmental conditions affecting many other taxa. Butterfly surveys were conducted over 16 months on an active military air reserve base located within a mosaic of densely populated urban, commercial, industrial, residential, and agricultural matrices in Homestead, southeast Florida. Butterfly species richness, abundance and diversity were documented, providing valuable base-line data for on-going butterfly monitoring, and the importance of this site's relatively healthy remnant ecosystems was evidenced by the supporting host plants for 20 migratory butterflies in 40 species. In addition, the air reserve base acts as refugia for many rare, endangered, and threatened federal and state-listed plants as well.

1. Introduction

Southeast Florida is a recognized 'hotspot' for biodiversity [1], home to endemic rare plants and animals found nowhere else in the world, many of which are located in globally endangered ecosystems such as globally endangered pine rocklands [2] and tropical hardwood hammocks [3]. Even as human-tolerant and still common wildlife such as raccoons and opossums are squeezed into decreasing suitable habitat fragments within urban matrices, the less-mobile, once-abundant species, including small invertebrates, may become extirpated or extinct [4-12]. Remaining islands of natural ecosystems are often infested with non-native, invasive plant and animal species which exacerbate the ability of native wildlife to survive [5,7,9,11-17]. In addition, these fragments of remnant

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habitat are usually surrounded by unsuitable, hostile environment with little safe connectivity between extant parcels [11,18-22]. The original pine rockland habitat, dominated by slash pines (*Pinus elliottii* var. *densa*) and rare endemic plants [23-26] has been reduced to less than 1.5% (Figure 1). Isolated safe haven areas (refugia) are often in danger of being lost to further commercial development, or agricultural areas, affecting population size, survival and dispersal capabilities [7,11,12,16,27-30].

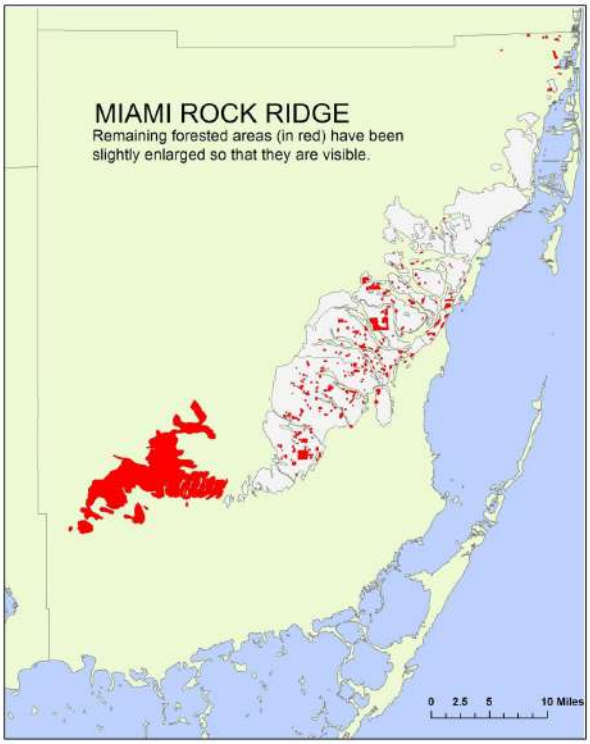

Figure 1. Remnant pine rockland areas remaining in Miami-Dade County are colored red; light gray outlines indicate original range

Source: Fairchild Tropical Botanic Garden.

Developed arenas invite increased use of pesticides and non-native vegetation, and vast agricultural monocultures not only use potentially harmful agrochemicals, insecticides and herbicides [15,31-35] but also heavy machinery that further degrades the soil structure and native vegetation (including removal of "weeds" that act as host plants for insect larvae and nectar sources for adult insects) [36]. In addition, South Florida agricultural lands consist of "rock-plowed" limestone outcrop, a process developed in the 1950's to make use of the substrate called "Miami oolite" [37] for agriculture. Many butterfly species that inhabit this unique environment are endemic to southeast Florida and are often rare or localized [8,22,38-40]. Four species are considered endangered by the United States Fish and Wildlife Service [41]: Bartram's scrub-hairstreak (*Strymon acis bartrami*), Schaus swallowtail (*Heraclides aristodemus ponceanus*), Florida leafwing (*Anaea troglodyta floridalis*) and Miami blue (*Cyclargus thomasi bethunebakeri*) [42]. Three additional species of butterflies in south Florida are protected by the Florida Wildlife Conservation Commission [43] and 26 are considered "imperiled" in south Florida by the State of Florida's Imperiled Butterfly Work Group, a subset of the Florida Wildlife Conservation Commission [43], which was formed in 2008 to study declining lepidoptera. Two species are now considered extinct, zestos skipper (*Epargyreus zestos oberon*) and Meske's rockland grass skipper (*Hesperia meskei pinocayo*) [43].

Surveys of butterfly populations have been used as an important monitoring protocol for ecological and biodiversity indicators for many years [12,39,44-48]. Our butterfly surveys show that the butterflies and plant species listed by the USFWS or FWC as endangered or threatened have safe refugia on the Homestead Air Reserve Base (HARB) (Figure 2); it is also valuable conservation data, strengthening long-term data collection that has been done in the Homestead area in nearby critical butterfly units [49] (Figure 3).

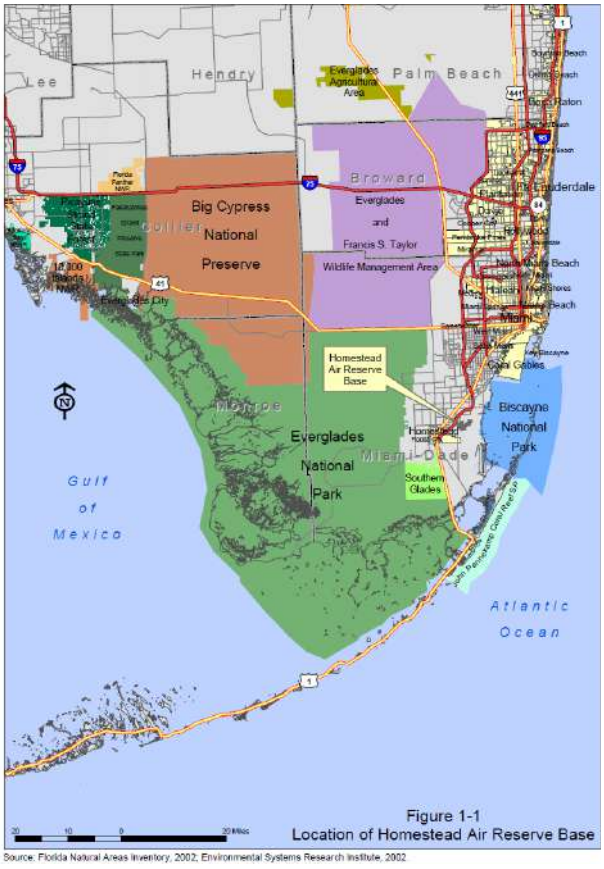

Figure 2. Homestead Air Reserve Base and surrounding matrices, located in southeast Florida

Source: Florida Natural Areas Inventory, 2002. Environmental Systems Research Institute 2002.

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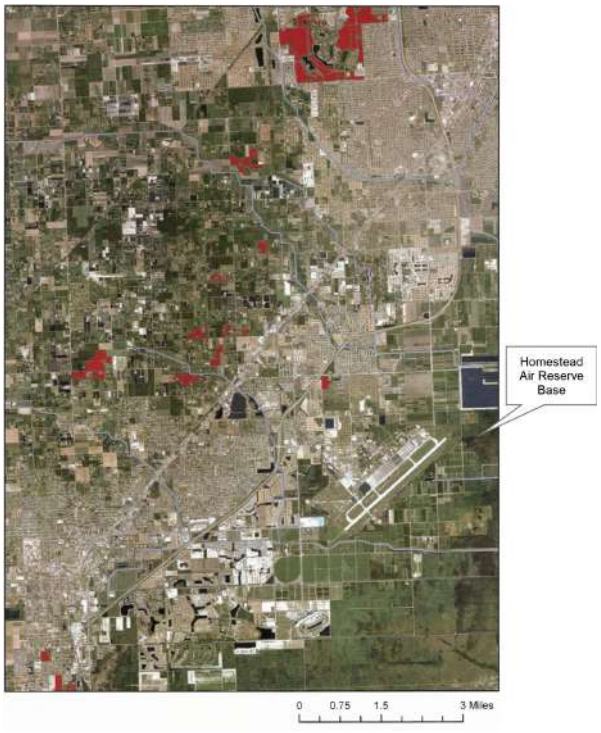

Figure 3. Critical butterfly habitat units (in red) located within proximity of Homestead Air Reserve Base

Source: M. Andrejko

2. Location and Description of Survey Units

Homestead Air Reserve Base (HARB) is an active military base located in southern Miami-Dade County, in Homestead, Florida (25° 29' 9.752", -80° 23' 27.298"). The base is located approximately 20 miles south-southwest of the city of Miami and about 1.5 miles inland from Biscayne National Park and the Atlantic Ocean (Figure 1). HARB is nestled within densely populated urban, commercial, industrial, residential, and agricultural matrices. The surrounding natural areas include globally endangered pine rockland fragments, fresh-water marshes containing low-lying herbaceous plants, coastal beaches with heavily used public and commercial boating facilities, tropical hardwood hammock fragments, mining operations, industrial complexes and derelict abandoned properties (Figure 2). Agricultural fields are the predominant landscape interspersed with residential, commercial and industrial facilities (such as limestone and sand mining). Critical butterfly habitat units, protected natural areas owned and maintained by Miami-Dade County, or in some cases by private landowners, are located south, west, and north of the airbase [40,50] (Figure 3).

HARB currently contains 1,943 acres; numerous ownership changes since the 1940's and a number of powerful hurricanes have caused significant damage over the years, reducing the original base acreage by nearly half. Large portions were sold or transferred as excess property after Hurricane Andrew in 1992 [51]. The areas within the base are divided into fourteen "Land Management Units" [51] (Table 1); 1,000 acres are described as "heavily modified pine rockland habitat" [51]. The airfield itself covers 945.3 acres, nearly half of the remaining land area. Most of the units were either off-limits for surveys because of concern for safety, such as ordnance storage, or because of military practices, such as training sessions or other air force activities. Some 'units' actually consist of parking lots and administration buildings and were therefore not suitable for butterfly occupancy. There is a mosaic of non-native vegetation, native endangered or threatened plants, grasslands, globally threatened pine rocklands, as well as old partially developed or damaged derelict areas. A lake and wetlands were not surveyed but are part of the off-limit areas to the southeast (runway area).

Table 1. Land management units identified on the base: (Integrated Natural Resources Management plan for Homestead Air Reserve Base, Homestead, Florida Volume 1, July 2009.)

Land Management Unit	Acreage
Boundary Canal	40,400 linear ft.
Administrative and Industrial Support	334.3 acres
Airfield Area	945.3 acres
Grenade Range and Reserves Area	116.6 acres
Hush House Area	30.6 acres
Munitions Area	122.0 acres
Northeast Grasslands	50.5 acres
Operable Unit (OU)-2 acre	21.1 acres
Phantom Lake (including old Grenade Range)	93.8 acres
Remnant Pine Rockland	5.1 acres
SOCSouth	14.0 acres
Southeast Triangle	51.9 acres
Southwest Clear Zone	57.0 acres
Twin Lakes and Wetland Fringe	40.8 acres
Wetland marsh	34.7 acres

3. Descriptions of Survey Areas

Pine Rockland Unit and Sample Site #7

On-going restoration at this northwest HARB pine rockland unit consisting of 5.1 acres was occurring as invasive non-native trees and exotic herbaceous vegetation were removed. The site contains mature and seedling Florida slash pine trees (*Pinus elliottii* var. *densa*), a species particular to south Miami-Dade and the Caribbean known for its extremely hard wood and high-leaved growth habit, due to being fire-adapted. The nutrient-poor limestone substrate in the rocklands has little soil or organic matter,

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and the pines grow slowly, making the wood dense and hard. Other native trees in the pineland area included Florida trema (*Trema micranthum*), the state-listed threatened West Indian lilac (*Miconia bicolor*) and the state tree, cabbage palm (*Sabal palmetto*). Herbaceous plants include the state-endangered pineland jacquemontia (*Jacquemontia curtsii*) and locustberry (*Byrsonima lucida*) (Figure 4).

Figure 4. Remnant pine rockland area; this site has more understory vegetation than regularly fire-maintained pine rockland areas (Copyright: Koi)

Sample Site #7, a small subset of approximately 2 acres located directly to the east of the pineland, consists of a few scattered mature and young Florida slash pines, and low-lying herbaceous native vegetation such as porterweed (*Stachytarpheta jamaicensis*), Spanish needle (*Bidens alba* var. *radiata*), and creeping ticktrefoil (*Desmodium incanum*). The federally endangered sandflax, (*Linum arenicola*), and the state-listed endangered quailberry (*Crossopetalum ilicifolium*) are also located here (Figure 5).

Figure 5. Sample site 7 contains a ruderal field with scattered non-native Australian pine seedlings (now destroyed) and native slash pine trees and seedlings. (Copyright: Abbott)

3.1 Boundary Canal Unit

The Boundary Canal unit consists of 40,400 linear feet (7.8 miles) of deep channels dug into the limestone substrate, which contain fresh water with high visibility except for a few areas polluted with urban trash that gets flushed into the canal from surrounding matrices. It harbors both native and non-native fish, and occasional ducks and wading birds, as well as numerous non-native reptiles. Fresh-water canals such as these are known to provide habitat for the Miami cave crayfish, *Procambus milleri*[52], a federally endangered species [43].

Weedy non-native tree seedlings and mature trees, primarily the exotic non-native Australian pine (*Casuarina equisetifolia*), line the edges of the canals. Native shrubs, such as myrsine (*Myrsine floridana*), and cocoplum (*Chrysobalanus icaco*), grow along the banks. Maintenance protocol at the base periodically sprays herbicide along the canal edges, causing temporary die-back of all vegetation. The Boundary Canal is shaded by the Australian pine trees and non-native Brazilian pepper (*Schinus terebinthifolius*) on the northwest and south routes and has grassy banks on the western route (Figure 6).

Figure 6. The southwestern edge of the boundary canal unit has a grassy bank with scattered trees along the canal, lined with non-native Australian pine and Brazilian-pepper on the eastern edge (Copyright: Abbott)

3.2 Grenade Range and Reserves Unit

A grenade range and reserves area, still used for military practices, consists of 116.6 acres of open mowed and un-mowed grasslands, and thin sand-soils lying on limestone outcrop. There is a rich variety of both native and non-native trees and vegetation. Native plants found include passionvine (*Passiflora suberosa*), porterweed, marlberry (*Ardisia escallonioides*), and sandflax (*Linum areniola*). There are sunny open areas of native and non-native grassy fields, both mowed and un-mowed. Native herbs in this unit include passionvine, Spanish needles, pencil-flowers (*Stylosanthes hamata*) and native milkweed (*As-*

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clepius suberosa), and the federally endangered Small's milkpea (*Galactia smallii*). There are also burned out vehicles and other blockades from military practices on this location, but we did not monitor near these artifacts. Non-native Australian pine and Brazilian pepper trees are mixed among native trees such as gumbo limbo (*Bursera simaruba*), poisonwood (*Metopium toxiferum*) and willow bustic (*Dipholis salicifolia*) (Figures 7 and 8).

Figure 7. The practice Grenade Range and Reserves Area consisted of mowed and unmown areas, as well as the derelict vehicles used in military training sessions (Copyright: Koi)

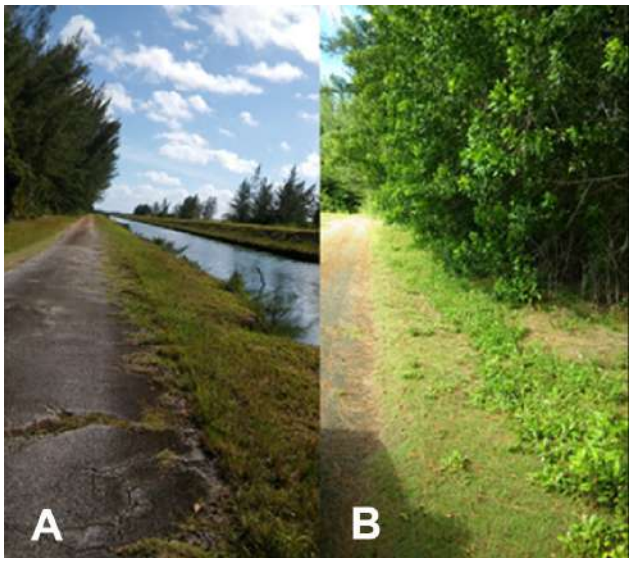

Figure 8. The edge of the Boundary Canal northwest perimeter has a narrow grassy edge, shaded by a mix of non-native Australian pine and Brazilian-pepper trees; the semi-shaded understory contained abundant weedy nectar and host plant resources. A. Facing the canal looking west. B. Facing the trees looking east (Copyright: Koi)

3.3 Special Operations South (SOCSouth)

This 14-acre area consists of a mosaic of remnant derelict road fragments, tropical hardwood hammock, restored pine rockland, and weedy un-mowed fields. It contains twenty-six federal- and state-listed trees such as silver palm (*Coccothrinax argentata*), and Bahama senna (*Senna mexicana* var. *chapmanii*). Some areas are densely overgrown and heavily shaded with native plants such as the semi-invasive jack-in-the-bush (*Chromolaena odorata*), an excellent nectar source for both butterflies and moths, as well as bees, beetles and other beneficial insects. Herbaceous vegetation such as the federally endangered sandflax, and Small's milkpea are found here as is the state-listed endangered pineland clustervine (*Jacquemontia curtsii*). The area has a wide variety of small native shrubs, including beautyberry (*Callicarpa americana*), saltbush (*Baccharis halimifolia*), native trees such as wild tamarind (*Lysiloma latisiliquum*) and royal palm (*Roystonea elata*). Florida state-listed ground-covering herbs such as wild potato morning glory (*Ipomoea microdactyla*), Bahama ladderbrake (*Pteris bahamensis*) and pineland lantana (*Lantana depressa*). On-going non-native plant removal also occurred at this location, to remove heavy infestation of Brazilian pepper and Burma reed (*Neyraudia reynaudiana*) (Figure 9).

Figure 9. Weedy un-mown fields surrounded by shrubs and slash pines dominate SOCSouth; scattered sections of old pavement are also located in this unit (Copyright: Koi)

4. Materials and Methods

Nine standard "Pollard Walk" [44] surveys were conducted in four diverse ecosystems located on the base to document butterfly species between 09-May-2015 and 30-December-2016. Pollard walks are used throughout the world to assess butterfly populations, and primarily consist of slowly walking along pre-determined set transects or routes in the chosen site, noting butterfly species within five meters on three sides (left, right and above). Care was taken to avoid double-counting as the insects flew and close-focus binoculars were used to identify species; species were photographed whenever possible. Walks sometimes had to be cancelled because of military exercises,

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heavy tropical storms, or restoration work, which accounts for the unevenness of the surveys.

Richness and diversity was determined using Shannon-Weiner Diversity Index, and Simpson Dominance Index, as well as the Equitability Index, and skewness was established via Kurtosis. Identification was done by an entomologist (one of the authors, SK), aided when necessary with photographic records from a university website [53] and a field guide [54].

5. Butterfly Survey Results

Forty species of butterflies were counted, in seven families, for a total of 2,128 individuals in the nine Pollard Walks (Table 2). We recorded 469 individuals in the pineland and Sample Site #7 (Figure 10), 1,425 individuals in the Canal and Grenade Range (Figure 11), and 234 individuals in SOCSouth (Figure 12). (Boundary Canal and Grenade Range were counted as one unit because they are contiguous in the actual landscape.)

Table 2. Butterflies observed at Homestead Air Reserve Baser by standard Pollard Walks from 9 May 2015 to 30 Dec. 2016, migratory status and host plants on base

Common Name	Scientific Name	Migratory	Hostplant(s)
Giant swallowtail	*Papilio cresphontes*	Yes	Wild Lime (*Zanthoxylum fagara*); Other citrus in surrounding matrix
Great southern white	*Ascia monuste*	Yes	Peppergrass *(Lepidium virginicum);* Other mustards in surrounding matrix
			Chapman's Wild Sensitive Plant *(Senna mexicana var. chapmanii)*
Pink-spot sulphur	*Aphrissa neleis*	Yes	Wild tamarind (*Lysiloma latisiliquum);* Sabicu (*L. sabicu)*
Cloudless sulphur	*Phoebis sennae*	Yes	Deering Partridge Pea *(Chamaecrista deeringiana);* Sensitive Pea *(C. nictitans var. aspera)*
Orange-barred sulphur	*Phoebis philea*	Yes	False Tamarind *(Lysiloma latisiliquum)*
Large orange sulphur	*Phoebis agarithe*	Yes	Beggarweed (*Desmodium incanum);* Threeflower Ticktrefoil *(D. triflorum)*
Little yellow	*Pyrisitia lisa*	Yes	Chapman's Wild Sensitive Plant (*Senna mexicana* var. *chapmanii*)
Barred yellow	*Eurema daira*	Yes	Partridge Pea *(Chamaecrista fasciculata);* Deering Partridge Pea *(C. deeringiana)*
Dainty sulphur	*Nathalis iole*	Yes	Spanish Needles *(Bidens alba* var. *radiata);* Cheesytoes *(Stylosanthes hamata)*
Martial scrub hairstreak	*Strymon martialis*	No	Florida trema (*Trema micanthrum*)
			Common Fanpetals *(Sida acuta);* Elliott's Fanpetals *(S. elliottii)*
Mallow scrub hairstreak	*Strymon istapa*	No	Sleepy Morning (*Waltheria indica*)
Fulvous hairstreak	*Electrostrymon angelia*	No	Brazilian-pepper (*Schinus terebinthifolius*)
Gray hairstreak	*Strymon melinus*	No	Common Fanpetals *(Sida acuta);* Elliott's Fanpetals *(S. elliottii)*
			Lead Tree (*Leucaena leucocephala*)
			Buttonwood (*Conocarpus erectus*); polyphagous*
Cassius blue	*Leptotes cassius*	No	Downy milkpea (*Galactia volubilis);* Small's Milkpea *(G. smallii)*
			False Tamarind *(Lysiloma latisiliquum)*
Ceraunus blue	*Hemiargus ceraunus*	No	Sensitive Pea (*Chamaecrista nictitans* var. *aspera);* Partridge Pea *(C. deeringiana)*
Zebra heliconian	*Heliconius charithonia*	No	Corkystem passionvine *(Passiflora suberosa)*
Julia heliconian	*Dryas iulia*	No	Corkystem passionvine *(Passiflora suberosa)*
Gulf fritillary	*Agraulis vanillae*	Yes	Corkystem passionvine *(Passiflora suberosa)*
Variegated fritillary	*Euptoieta claudia*	Yes	Sand Flax (*Linum arenicola*); Everglades Flax *(L. carteri)*
Pearl crescent	*Phyciodes tharos*	No	Scaleleaf Aster *(Symphyotrichum adnatum)*
Phaon crescent	*Phyciodes phaon*	Yes	Turkey Tangle Fogfruit *(Phyla nodiflora);* Southern Fogfruit *(P. stoechadifolia)*
Buckeye, tropical	*Junonia zonalis (=genoveva)*	No	Blue Porterweed *(Stachytarpheta jamaicensis)*
Buckeye, common	*Junonia coenia*	Yes	False Foxglove *(Agalinis fasciculata)*; Sand Flax (*Linum arenicola)* (?)**
White peacock	*Anartia jatrophe*	Semi	Turkey Tangle Fogfruit *(Phyla nodiflora);* Southern Fogfruit *(P. stoechadifolia)*
			Herb-of-Grace *(Bacopa monnieri)*
Ruddy daggerwing	*Marpesia petreus*	Yes	Strangler fig (*Ficus aurea);* Laurel Fig *(F. microcarpa)*
Monarch	*Danaus plexippus*	Yes	Scarlet Milkweed *(Asclepias* curassavica*)*
Queen	*Danaus gilippus*	Semi	Scarlet Milkweed *(Asclepias* curassavica*)*
Long-tailed skipper	*Urbanus proteus*	Yes	Beggarweed (*Desmodium incanum);* Threeflower Ticktrefoil *(D. triflorum);* polyphagous*
Dorantes longtail	*Urbanus dorantes*	Semi	Beggarweed (*Desmodium incanum);* Threeflower Ticktrefoil *(D. triflorum);* polyphagous*
Horace's duskywing	*Erynnis horatius*	No	Oak species (*Quercus* sp.)
Tropical checkered skipper	*Pyrgus oileus*	No	Common Fanpetals *(Sida acuta);* Elliott's Fanpetals *(S. elliottii)*
Neamathla skipper	*Nastra neamathla*	No	Bluestem grass (*Andropogon* sp.)

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Three-spotted skipper	*Cymaenes tripuncta*	No	Guinea Grass (*Panicum maximum)*
Clouded skipper	*Lerema accius*	Yes	Saint Augustine Grass *(Stenotaphrum secundatum)*
Southern skipperling	*Copaeodes minimus*	No	*Bermuda grass (Cynoden dactylon)*
Least skipper	*Ancyloxypha numitor*	No	Giant Cutgrass *(Zizaniopsis miliacea)*
Sachem skipper	*Atalopedes campestris*	Yes	*Bermuda grass (Cynoden dactylon);* Saint Augustine Grass *(Stenotaphrum secundatum)*
Fiery skipper	*Hylephila phyleus*	Yes	*Bermuda grass (Cynoden dactylon);* Saint Augustine Grass *(Stenotaphrum secundatum)*
Baracoa skipper	*Polites baracoa*	No	Grass sp.; polyphagous*
Southern broken-dash	*Wallengrenia otho*	No	Saint Augustine Grass *(Stenotaphrum secundatum);* Thin paspalum *(Paspalum setaceum)*

Notes: *Polyphagous refers to a wide variety of food plants; most common host plants are listed but these may not be the only host plants used by the species. **Lineria is the usual hostplant; Linum may be as well based on chemical composition comparisons[79-81].

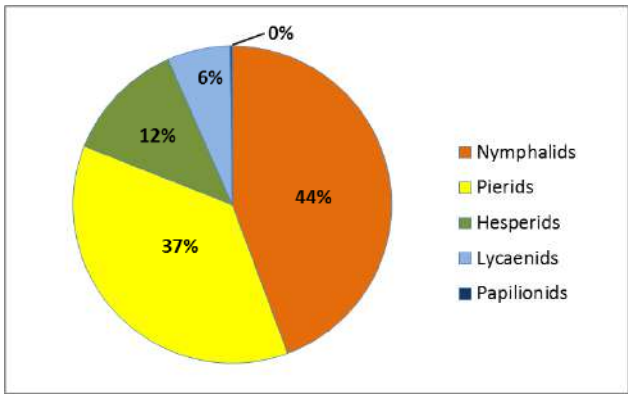

Figure 10. Pine Rockland Unit and Sample Area #7 were dominated by Nymphalids and Pierids, with a few Lycaenids and only one sighting of a Papilionid, which accounted for less than 0.002% of the total individuals observed and is represented by 0% on the graph

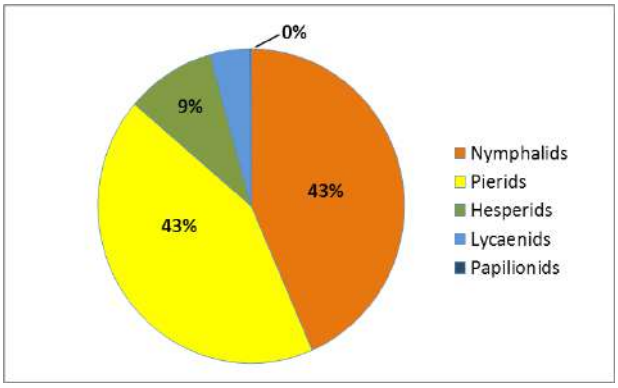

Figure 11. Species distribution in the practice Grenade Range and Boundary Canal Unit was dominated by the Nymphalid and Pierid families

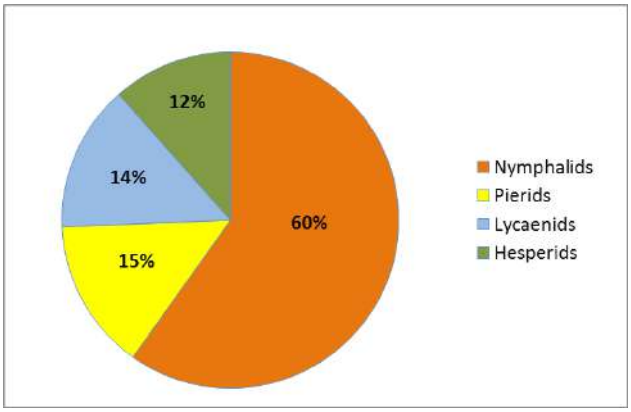

Figure 12. SOCSouth butterfly distribution was dominated by butterflies in the Nymphalid family and had more Lycaenids than any other habitat on the base

The least represented species were swallowtails (Papilionid family), with only three individuals in one species, the giant swallowtail (*Papilio cresphontes*), which was also only seen in one count (Figure 13). There were seven species and 816 individuals of "whites and yellows" (Pierid family) counted, the most abundant being barred yellow (*Eurema daira*), which accounted for 79% of the Pierids and 30% of all species. Dainty sulphurs (*Nathalis iole*) were the second most abundant in the family, accounting for 9% of all of the Pierids. Other species observed were great southern white (*Ascia monuste*), large orange sulphur (*Phoebis agarithe*), little yellow (*Eurema lisa*), and orange-barred sulphur (Phoebis philea) (Figure 13).

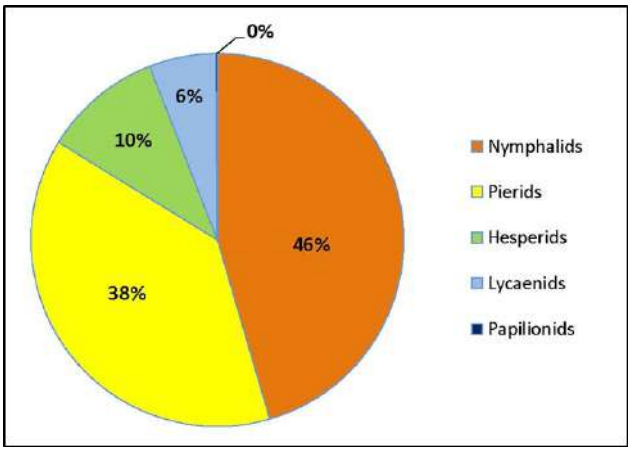

Figure 13. All butterfly families and abundance documented at Homestead Air Reserve Base counted by standard Pollard Walks from 9 May 2015 to 30 Dec. 2016. The Papilionid count consisted of only three individuals, which was less than 0.001% of the total individuals observed, statistically counted as "0%"

The very small butterflies known as "blues" accounted for 4% of all butterflies counted, in only two species, ceraunus blue (*Hemiargus ceraunus*) and cassius blue (*Lep-*

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totes cassius). Although both species are common, both butterflies are listed as endangered by the federal government [42], because of their similarity to the federally endangered Miami blue (*Cyclargus thomasi bethunebakerii*) [42]. Ceraunus blue was more common than cassius blue (Figure 13).

Only four species of "hairstreak" butterflies (Lycaenidae family), named for the tiny tail-like protrusions on their hindwings, were seen. Numbering just 20 individuals, they accounted for less than 1% of the butterfly species recorded. The most observed species was fulvous hairstreak (*Electrostrymon angelia*). There was only one each observed of mallow scrub-hairstreak (*Strymon istapa*), martial scrub-hairstreak (*Strymon martialis*), and gray hairstreak (*Strymon melinus*) (Figure 13).

The "Brushfoot" butterflies (Nymphalids) were the most abundant family observed at each survey, totaling 933 individuals in nine species. Gulf fritillary (*Agraulis vanillae*) butterflies accounted for 46% of all Nymphalids observed, and 34% of all butterflies observed, with 433 individuals. White peacocks (*Anartia jatrophae*) were the second most abundant.

We did not differentiate between the two possible Southeast Florida buckeye butterflies tropical buckeye (*Junonia zonalis*) and common buckeye (*J. coenia*) (see detailed note and citations regarding *Junonia* species and migration). Because buckeyes scatter quickly when approached, it was challenging to verify species consistently in the field. The majority were likely "tropical" as opposed to "common" because the host plant for the tropical buckeye, blue porterweed (*Stachytarpheta jamaicensis*), is abundant on the property. Tropical buckeyes do not migrate, but common buckeye does. However, they hybridize in locations where the migratory route of the common buckeye overlaps with the resident populations of the tropical species. For this reason, 'buckeye' species were counted as one group. (The third species, mangrove buckeyes (*J. evarete*) are not found on the base as there are no host plant mangroves on the surveyed property and this species does not migrate).

Zebra heliconian (*Heliconius charithonia*) was the next abundant species seen, and we also observed variegated fritillary (*Euptoieta claudia*), phaon crescent (*Phyciodes phaon*), julia heliconian (*Dryas iulia*), pearl crescent (*Phyciodes tharos*) and ruddy daggerwing (*Marpesia petreus*) (Figure13).

There were 46 individuals counted in the milkweed butterfly family (Danaid), which are Nymphalids, 25 queens and 23 monarchs (Figure 13). No soldiers, the third Danaid species, were observed on base (pun intended)!

Skipper butterflies (Hesperidae family) were much less abundant than expected given the profusion and diversity of graminoid species in the grassy fields on the air base, as many skippers are grass feeders. Fourteen species were observed, totaling 199 individuals. The most abundant species was baracoa skipper (*Polites Baracoa*), representing 31% of all skippers observed. Longtailed skippers (*Urbanus proteus*) were the next abundant, followed by the Dorantes skipper (*Urbanus dorantes*). We also recorded fiery skipper (*Hylephila phyleus*), clouded skipper (*Lerema accius*), three-spotted skipper (*Cymaenes tripunctus*), tropical checkered skipper (*Pyrgus oileus*), monk skipper (*Asbolis capucinus*), ocola skipper (*Panoquina ocola*), sachem skipper (*Atalopedes campestris*), southern broken-dash skipper (*Wallengrenia otho*), southern skipperling (*Copaeodes minimus*), least skipper (*Ancyloxypha numitor*), neamathla skipper (*Nastra neamathla*), and palmetto skipper (*Euphyes arpa*) (Figure 13).

Shannon Diversity Index shows fairly high diversity (2.554) dominated by a few species (Simpson's Index of Dominance: 0.8612). The species evenness was low (0.692), and the skewness/Kurtosis was high (3.697/14.308).

6. Discussion

Tracks of land protected from further development and alteration, such as HARB and other military and reserve bases, act as vital refugia for plant and animal taxa, as well as providing connectivity between remaining natural areas, even in heavily urbanized surrounding matrices [30,49,55]. However, the natural areas within these agricultural-urbanized matrices may 'suffer from detrimental edge effects' [5-7,11,50,56], primarily because the natural 'edges' or ecotones no longer exist and the biotic taxa that may have lived within the transition zone between ecosystems cannot adapt to the changes between a natural area and an artificial matrix. Butterflies and other insects often require specific elements in 'landscape architecture' as well as host plants and nectar resources in order to persist in a location [20,57]. In addition, fire-maintained ecosystems such as pine rocklands suffer from fire suppression in urban areas [50].

There was a pronounced difference in the abundance of butterflies in the different units (Table 3). The highest number of species (34) and most individuals (1424) counted was in the Practice Grenade Range/Boundary Canal Unit, which had the most habitat diversity and greatest acreage. Weedy, overgrown, limestone outcrop areas such as the Pine Rockland and Sample Area 7 units hosted thirty species and we counted 469 individuals there. The lowest species count (29) and lower number of individuals

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(243) counted was in the SOCSouth Unit, which at that time was still undergoing restoration efforts.

Table 3. Butterfly species abundance as observed in the units surveyed on Homestead Air Reserve Base

Common Name	Scientific Name	Pine Rockland/ Sample Area 7	SOC-South	Practice Grenade Range/ Boundary Canal
Giant swallowtail	*Papilio cresphontes*	1	-	2
Great southern white	*Ascia monuste*	2	-	24
Cloudless sulphur	*Phoebis sennae*	8	7	29
Orange-barred sulphur	*Phoebis philea*	1	4	-
Large orange sulphur	*Phoebis agarithe*	1	4	7
Little yellow	*Pyrisitia lisa*	2	7	2
Barred yellow	*Eurema daira*	149	10	487
Dainty sulphur	*Nathalis iole*	9	2	60
Martial scrub-hairstreak	*Strymon martialis*	-	-	1
Mallow scrub-hairstreak	*Strymon istapa*	4	-	2
Fulvous hairstreak	*Electrostrymon angelia*	-	13	-
Gray hairstreak	*Strymon melinus*	-	1	-
Cassius blue	*Leptotes cassius*	1	8	18
Ceraunus blue	*Hemiargus ceraunus*	25	11	39
Zebra heliconian	*Heliconius charithonia*	4	6	53
Julia heliconian	*Dryas iulia*	2	-	7
Gulf fritillary	*Agraulis vanillae*	101	58	261
Variegated fritillary	*Euptoieta claudia*	11	18	3
Pearl crescent	*Phyciodes tharos*	-	3	1
Phaon crescent	*Phyciodes phaon*	3	1	29
Buckeye species	*Junonia* sp.	67	32	32
White peacock	*Anartia jatrophe*	12	7	203
Ruddy daggerwing	*Marpesia petreus*	-	2	1
Monarch	*Danaus plexippus*	2	3	18
Queen	*Danaus gilippus*	6	10	11
Long-tailed skipper	*Urbanus proteus*	1	6	48
Dorantes longtail	*Urbanus dorantes*	-	3	20
Tropical checkered-skipper	*Pyrgus oileus*	-	-	6
Neamathla skipper	*Nastra neamathla*	-	-	1
Three-spotted skipper	*Cymaenes tripunctus*	1	1	9
Clouded skipper	*Lerema accius*	6	-	5
Southern skipperling	*Copaeodes minimus*	1	-	1
Least skipper	*Ancyloxypha numitor*	2	-	7
Sachem skipper	*Atalopedes campestris*	-	4	1
Fiery skipper	*Hylephila phyleus*	8	1	4
Baracoa skipper	*Polites baracoa*	32	3	30
Southern broken-dash	*Wallengrenia otho*	2	2	-
Palmetto skipper	*Euphyes arpa*	1	-	-
Monk skipper	*Asbolis capucinus*	4	1	2
Ocola skipper	*Panoquina ocola*	-	6	-
Total abundance		469	234	1424
Total species		30	29	34

While many land managers must think in big pictures (saving biomes for bears or whales), the micro-scale at which many insects live is something often overlooked: even small tracts of land, if providing necessary elements for survival, may be important for the persistence of rare and endangered endemic taxa [17,19,20,22,30,49,56,57]. Fire-driven and fire-adapted pine rockland habitats may contain as many as 536 plant taxa [37,58,59] and even small isolated fragments may contain exceeding rare or threatened species [38,59,60]. This is true for the HARB pine rockland unit, which contains four Florida state endangered plants, and over fifty native plants. Only nine non-native plants were found in the small pineland, which have since been eradicated.

The hostplants (larval food plants) for all butterfly species observed are found on the base, with the exception of the recorded food for the palmetto skipper, saw palmetto (*Serenoa repens*), (Table 2). It is possible that the palmetto skipper is utilizing the other palm species located on the base, or saw palmetto located in nearby critical butterfly units (Figure 3). A wide variety of nectar sources is also site, providing continuous bloom[59,60] appropriate for the adults of all species (generally, small butterflies need small flowers and large butterflies need large flowers). Homestead is also a "low-income" community (per capita income is below $18K) [61], and there are many 'less-manicured' homes in the residential area,

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which is beneficial for weedy overgrowth of nectar and host plants in the surrounding matrix. Although the agricultural fields surrounding the base may provide some additional host and nectar sources, pesticide and herbicide use is also frequent [6,35,50,62]. Florida has a year-round growing season.

HARB is located on the eastern coast of south Florida, along the "Atlantic Flyway," where birds, dragonflies and butterflies follow the trade winds to aid in migrating south or north, depending on season, increasing its value as a stop-over site and refugia. One author (SK) witnessed mass migrations of checkered whites (*Pontia protodice*) and great southern whites flying through southern Homestead, ovipositing and mating on the abundant peppergrass (*Lepidium virginicum*) growing along the edges of the agricultural fields, and then saw the farm workers come out soon after to spray pesticide-herbicide to kill any and all butterflies, eggs and larvae. Simply converting to agricultural fields from original habitats is destructive to most butterfly assemblages, especially in tropical and neo-tropical regions[11,39,56]. The toxins from these anthropogenic chemicals are detrimental to development and survival of insect pest species in agricultural fields, but also affect non-target butterflies and beneficial insects [6,31-35,39,62].

Of the 40 butterfly species observed, half (50%) are long-distance migratory or at least exhibit a short distance but wide dispersal [63,64] (Table 2). Besides the well-known monarchs, great southern and checkered whites, and gulf fritillaries (so named because they migrate across the Gulf of Mexico), also migrate in swarms. Other butterflies migrate, but not long-distance, and some, such as the dainty sulphur, may disperse as far north as New York. Barred yellow, dainty sulphur, great southern whites, gulf fritillaries also exhibit periodic outbreaks or eruptions of large numbers of individuals, often during migratory or dispersal events [63,64].

The giant swallowtails were seen in May, when the citrus trees were in heavy flower and new foliar growth. Three or more broods are known to occur in south Florida, and it is odd that we observed only these three swallowtails and no other swallowtail species. It may be that our surveys missed the main dispersal events for these butterflies.

The relative abundance of fulvous hairstreaks may be attributed to the remaining regrowth of its only host plant, the Brazilian pepper tree, one of the most aggressive non-native species on the base. The tree is ubiquitous throughout South Florida, even in areas that have undergone restoration work. A Caribbean species, fulvous hairstreak was first recorded in Florida in the early 1970's, arriving by unknown means; it is a rare example of a naturalized butterfly species that has benefitted from non-native plants. Invasive plants and animals have been associated with decreases in native species regardless of the ecosystems involved and non-native predators may also influence the survival of adults and offspring [8,10-17,50,65-67].

It is also noteworthy to remember that less-optimal sites may act as "sinks" for some butterfly species, i.e., the primary host plant may not be available, so a female butterfly may choose inferior plants because that is all from which she has to choose at a remaining site [15,16,28-30,68]. In this scenario, the offspring may not survive, or may survive but not thrive, on the inferior or alternate host, causing the offspring to develop various inbred genetic faults or weakened immune systems, and/or the surviving adults may not be robust enough to mate and perpetuate the colony [15,19,27,29,30,68,69] Because the butterflies are actually occupying a site is not always an indication that the site is "high-quality habitat"; it may be all that is left for them [9,11,16,17,30,67,68].

For most butterfly species, we do not have the population viability analysis (PVA), which is how to determine if a colony is large enough to prevent collapse through genetic failure or food resource loss [30] and can calculate colony persistence for most animals. But what has been fairly well established is that habitat protection is of more importance and more telling for species survival than PVAs, because butterflies do not have the same kind of life history as mammals and avian species [30,40]. For example, Longcore and Osbourne [30] point out that a "molehill" may indeed be a "mountain" for animals that are the size of a dime or a quarter! Small urban preserves are increasingly valuable for the survival of rare plants and insects [16,17,30,40,49,66-68] and the risk of extinction increases with isolation from other sites [5,12,16,38,66] as well as connectivity between habitat patches and the surrounding matrix of those patches [6,16-18,66,70].

We were surprised that we did not see the federally endangered Bartram's scrub-hairstreak, or Florida leafwing butterflies on the base, as there are thousands of their sole host plant, pineland croton (*Croton linearis*) on the surveyed sites. One author (SK) spoke to retired HARB military personnel at a butterfly conference in 2015 who mentioned that Bartram's used to be present on the airbase during the 1950's and 60's. Possible reasons for extirpation of the butterflies include the increasing isolation of this site from the other 'critical butterfly units' and pine rockland fragments in Homestead and south Miami-Dade County, because of increasing development of the surrounding matrix. In addition, HARB was heavily damaged

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in Hurricane Andrew in 1992, and the presence of exotic animals and plants has exploded since then; there has been inconsistent maintenance over the years because of funding cuts and the absence of fire, as well as a number of smaller but damaging hurricanes.

Not all butterfly species are able to adapt to living in urban environments, although a few have managed to carve out a niche in domestic gardens and botanical reserves; the atala butterfly (*Eumaeus atala*), a former denizen of the disappearing pine rocklands, has made itself quite at home in private gardens since the 1980's with human assisted re-location programs [17,21,66,67,70,71]. It is unlikely that either Bartram's scrub-hairstreak or the Florida leafwing will take to living in metropolitan backyards, but one can hope that such an event will take place should they be provided with sufficient host plant, landscape architecture, and protection from urban pesticide use. In the meantime, HARB is acting as an important stop-over point and permanent refugia for butterflies and other migratory insects, such as dragonflies.

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Note on *Junonia* species

We use the new species determination for the tropical buckeye as *Junonia zonalis*, according to genetic analysis of the genus by Lalonde[72-74]. In the recent past, Turner and Parnell[75] named the tropical buckeye as *Junonia genoveva* and the Mangrove Buckeye as *Junonia evarete*. There were no type specimens from the 1700's, only color illustrations in books published by Cramer[76]. Recent observations by Neild [77] reversed these names, but most scientists and older field guides stay with the descriptions made by Turner and Parnell[75]. Lalonde's seminal genetic work on the buckeye phylogeny is very valuable. It is known that the *Junonia* hybridize in South Florida and possibly elsewhere, complicating identification [72-74,76,78].

Declarations

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Conflicts of Interest

The authors proclaim there are no conflicts of interest.

Availability of Data and Material

All data and material are available via the Homestead Air Reserve Base archives or the Institute for Regional Conservation with Department of Defense release.

Code Availability

Not applicable.

Authors' Contributions

CVDH directed the study, SK designed and executed the surveys, CVDH and SK analyzed the data, SK wrote the manuscript and CVDH provided editorial advice.

Ethics Approval

On behalf of, and having obtained permission from my co-author, I declare that:

(1) the material has not been published in whole or in part elsewhere except as an original biological report to the Homestead Air Reserve Base, Homestead, Florida;

(2) the paper is not currently being considered for publication elsewhere;

(3) both authors have been personally and actively involved in substantive work leading to the report, and will hold themselves jointly and individually responsible for its content;

(4) all relevant ethical safeguards have been met in relation to patient or subject protection, or animal experimentation.

Consent to Participate

Not applicable.

Consent for Publication

Permission to publish was granted by Timothy F. Norton, 482d Fighter Wing Public Affairs, Homestead Air Reserve Base, Homestead FL, on 11 Nov 2019. (786) 415-7330

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